Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 64 JANUARY, 1947 No. 1

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15679

Relation of Survival Time of Respiratory Gasping Mechanism of the Isolated Mouse Head to Age.

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It was shown previously[1] that survival of the "primitive respiratory center" of the rat head is inversely proportional to age of the animal. The method used was the recording of the gasping (mandibular movements) following decapitation. Young rats (under 5 or 6 weeks of age) showed 2 periods of gasping, the initial period being aerobic and the second period anaerobic. Rats older than 5 or 6 weeks were reported to show only the first (aerobic) series. Similar results were later obtained with ischemic heads of rabbits, cats, and dogs.[2] Work from our laboratory[3] has corroborated these findings, however it has been observed[4] that occasionally an adult rat may show a few gasps of the second series. Kabat[5] previously demonstrated a reciprocal relationship between age and resistance to arrest of brain circulation in dogs.

That the sudden change of the newborn mammal from the fetal to the outside environment may be more successfully accomplished a source of anaerobic energy is available to the young. This fact coupled with

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[1] Selle, W. A., and Witten, T. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 495.

[2] Selle, W. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 417.

[3] Hiestand, W. A., Tschirgi, R. D., and Miller, H. R., *Am. J. Physiol.*, 1944, **142**, 153.

[4] Tschirgi, R. D., personal communication.

[5] Kabat, J., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 534.

TABLE I.

Showing Relation of Age to Number of Gasps and Survival Time in 144 White Mice.

Age in days	1	3	4	5	6	7	8	9	10	11	12	15	19	over 20
No. mice used	8	6	6	6	6	7	10	7	7	6	11	2	13	49
Wt in g (avg)	1.7	2.3	2.4	2.7	3.1	3.5	2.6	2.3	4.9	5.3	4.7	4.6	9.3	27.5
No, gasps 1st ser. (avg)	12.5	9.5	9.8	8.7	8.1	8.1	6.0	11.3	6.4	3.7	6.4	8.0	7.2	10.9
No. gasps total (avg)	32.1	28.2	20.9	20.3	21.3	26.7	45.7	22.7	43.4	21.5	16.8	22.0	7.2	10.9
Duration of 1st ser. in sec. (avg)	32.2	19.0	14.5	25.5	13.3	13.1	19.5	24.6	11.6	10.1	13.3	14.5	18.6	16.05
±S.E.	±2.2	±1.5	±2.5	±4.0	± .97	± .04	±2.5	±2.9	± .68	± .99	±1.1	±3.6	±1.8	± .25
Total survival time in sec. (avg)	1652.1	1459.2	1326.3	780.0	916.0	576.1	574.3	754.3	354.3	109.0	71.8	64.5	19.9	16.05
±S.E.	±135.4	±101.3	±119.6	±92.5	±67.1	±62.7	±127.7	±46.5	±32.8	±12.1	±10.3	±35.0	±1.8	± .25

the ability of the young to withstand anoxia more effectively than the adult has been amply demonstrated.[6-10]

In the present investigation 144 white mice of the Purdue Swiss and Hygienic strains were used, no appreciable difference appearing in the gasping patterns of the 2 strains. Heads were decapitated with a single stroke of a razor blade held vertically in a holder as previously used with rats.[3] No gasping results in the mouse unless the cut is made posterior to the fore-legs. Rats will gasp if the cut occurs posterior to the external ear lobe. In both cases the excised head con-

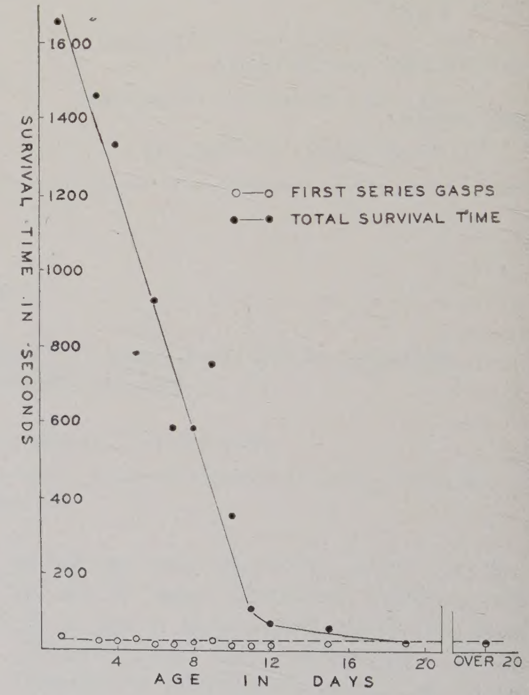

FIG. 1.

Showing survival time in relation to age in 144 mice. Each point represents the average of mice used at a given time.

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[6] Avery, R. C., and Johlin, J. M., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 1184.

[7] Fazekas, J. F., Alexander, F. A. D., and Himwich, H. E., *Am. J. Physiol.*, 1941, **134**, 281.

[8] Kabat, H., *Am. J. Physiol.*, 1940, **130**, 588.

[9] Himwich, H. E., Fazekas, J. F., and Homburger, E., *Endocrinol.*, 1943, **33**, 96.

[10] Himwich, H. E., Bernstein, A. O., Herrlich, H., Chesler, A., and Fazekas, J. F., *Am. J. Physiol.*, 1942, **135**, 387.

tains the medullary centers as well as the carotid reflex areas. Gasps were recorded manually on an electric kymograph traveling one centimeter in 5.540 seconds. Ninety-five mice from the ages of 1 to 19 days and 49 mice older than 20 days were used. In no case did any gasping occur beyond the first (aerobic) series in mice over 19 days of age. This observation holds true for many other mice used in other experiments and not included herein.

Table I and Fig. 1 show the results of this study. It can be seen that the aerobic first series of gasping remains quite constant throughout the life of the mouse indicating that the availability of the energy material remains little changed. The second or anaerobic series of gasps diminishes until about the 19th day when it is lost forever, thus indicating that the anaerobic energy of the newborn mouse persists for only 19 days.

Conclusions. The survival of the gasping mechanism of decapitated mice, like rats, is inversely related to age. Mice up to 19 days of age show 2 series of gasps, the early or aerobic series remaining quite constant throughout life, the second or anaerobic series gradually diminishing from the maximum in the newborn until it disappears completely at about 19 days of age.

15680

Effects of Urethane (Ethyl Carbamate) on Mitosis.*

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The authors[1] showed that transplanted rat carcinoma (Flexner-Jobling carcinoma) was far more susceptible to the lethal effects of X-rays when growing on rats which had been treated with colchicine than when growing on untreated controls. Unable because of war conditions to obtain colchicine to continue their experiments, they resorted to urethane (ethyl carbamate, $C_2H_5OCONH_2$) which was reputed to have much the same effects on mitosis. They found that while urethane does affect mitosis, the result was not the same as that following colchicine. The present study deals with their experiments and observations on this point.

The treatments were standardized to intraperitoneal injections of 1 cc of a 10% aqueous solution of urethane per 100 g of body weight of the young adult rats being treated. This was an anaesthetizing dose which left the animals inert for several hours. Of the 42 animals treated, 21 were male and 21 female. No significant sexual differences were found. A series of 14 mice were also injected and studied for comparison.

Since the purpose of our study was to determine the effect of urethane on mitosis it was important to select a tissue favorable for study. After some exploration we settled upon the corneal epithelium, recommended by Buschke, Friedenwald and Fleischmann[2] for mitosis counts and for differentiation of the various phases of mitosis. While the number of division figures vary greatly from the periphery to the center of the cornea, they are approximately the same in all meridians. A band 2 mm wide was cut out along the greatest meridian of the cornea and carefully separated from adherent tissues. After rinsing in 80% alcohol it was placed for an hour each in 95% alcohol, and absolute alcohol, and then stored in cedar oil. After at least an hour in cedar

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* Aided by support from McArdle Memorial Laboratory, the Wisconsin Alumni Research Foundation, and the Brittingham Trust Fund.

[1] Guyer, M. F., and Claus, P. E., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 565.

[2] Buschke, W., Friedenwald, J. S., and Fleischmann, W., *Bull. Johns Hopkins Hosp.*, 1943, **72-73**, 144.

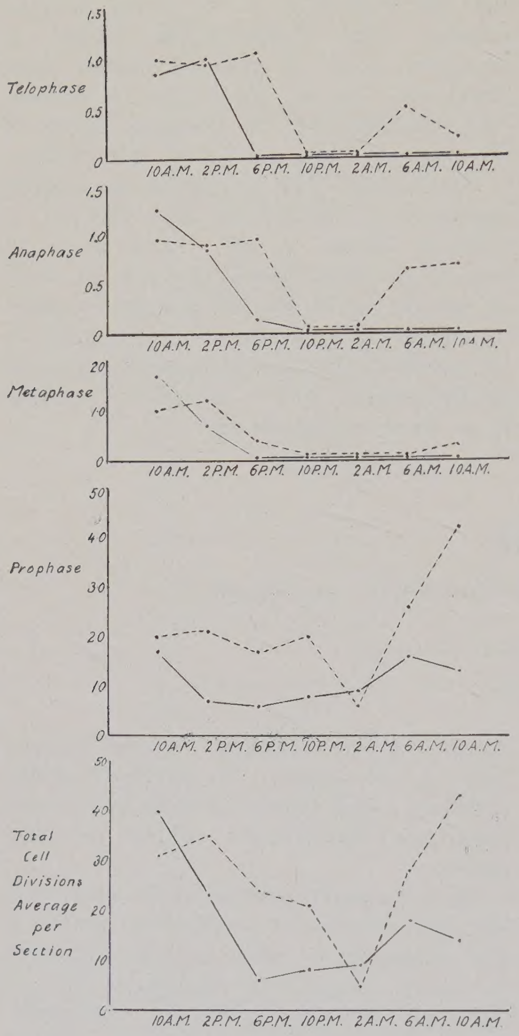

FIG. 1.

Graphs based on counts from the corneal cells of 42 female rats, showing the relative frequencies of the several mitotic stages in individuals killed at 4-hour intervals from 10 a. m. to 10 a. m. The continuous lines indicate mitotic phases in the 21 urethane-treated animals; the broken lines, the phases in the 21 controls. Counts for each phase in both experimental and control animals were made on 3 different individuals. The lowermost series represents the general average for all stages together.

oil the strips were passed successively through absolute alcohol, 95% alcohol, 70% alcohol, and distilled water, remaining for at least 15 minutes in each. They were then stained for 20 minutes in a 1:5 dilution of Harris' modification of Delafield's hematoxylin, rinsed in distilled water, differentiated in 1% HCl until deep red, and then left in ammonia water (0.1%) until blue. No counterstain was used. The strips were then dehydrated in the usual manner and cleared in cedar oil.

For sectioning, the tissues were mounted in paraffin and cut 6 μ thick meridionally of the cornea. According to the study of Buschke, Friedenwald and Fleischmann already cited, one in every 250 cells of the 2 basal layers should, on the average, be found in mitosis. While the range from eye to eye of the same individual is small the variation from animal to animal may be considerable.

The action of urethane on cell-division does not resemble closely that of colchicine. Although delaying mitosis for from 15 to 25 hours, colchicine permits accumulation and persistence of the metaphase stage, with actual continuance of chromosome-divisions. The interference appears to be mainly with

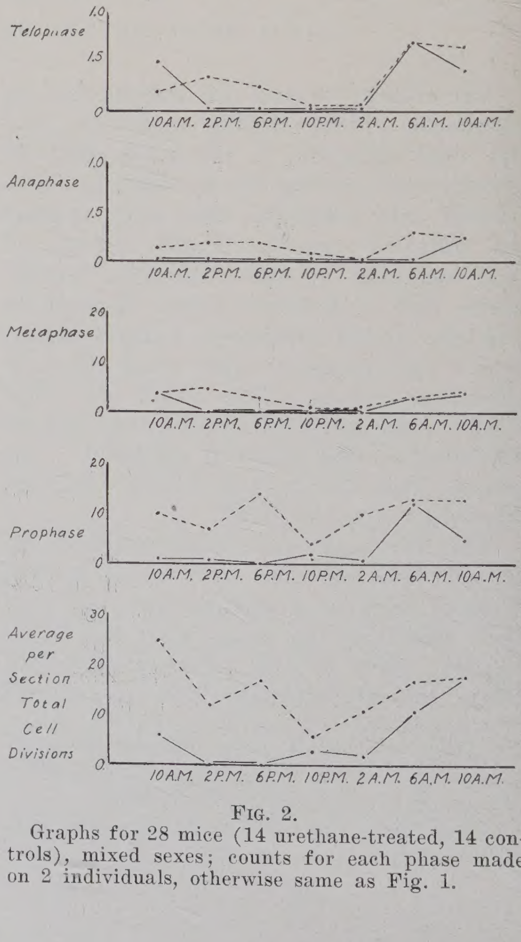

FIG. 2.

Graphs for 28 mice (14 urethane-treated, 14 controls), mixed sexes; counts for each phase made on 2 individuals, otherwise same as Fig. 1.

the distributional mechanism. With urethane, on the contrary, all mitotic stages seem to be largely done away with. Fig. 1 and 2 show how nearly mitotic activities were brought to a standstill.

The graphs are based on corneal sections in which mitotic figures of all phases were counted in every fifth section, with 36 sections inspected for each cornea. Forty-two rats were injected at 9:30 a. m. after which 6 injected and 6 control animals (3 of each sex) were killed at intervals of 4 hours from 10 a. m. one morning till 10 a. m. the next—a sequence of 7 sets in all. For purposes of comparison similar counts were also made on 28 mice.

Fig. 1 represents a series of graphs for the average abundance of each stage, based on sections from the corneas the 21 urethane-treated female rats compared with 21 corneal mitotic counts from untreated controls. The lowermost graph summarizes the general average for all stages taken together. The continuous line of each graph represents the urethane-treated animals, the dotted line, the controls. Horizontal readings indicate the time of day the animals were killed, the upright column shows the average number of division-figures per section in 108 sections (3 × 36) of the 3 females killed at any one time. The averages for anaphases and telophase (the 2 uppermost graphs) are based upon a 0 to 1.0 scale in contrast with the others in which the scale ranges from 0 to 50. This is because so many fewer division figures are found in anaphase and telophase than in prophase and metaphase.

The controls, indicated by dotted lines, show a daily rise and fall of the mitotic activities of all stages, with the smallest number of division figures appearing at or a little before 2 a. m., after which it ordinarily rises fairly rapidly till at least 6 a. m. In the treated animals, indicated by the solid lines in the graphs, the disappearance of the several stages is more rapid and it reaches its lowest point several hours earlier than the normal—by approximately 6 p. m. After remaining stationary or markedly retarded for from 8 to 12 hours, prophases begin to increase in number again. A series of counts on the corneas of the 21 injected and 21 control male rats was also made but inasmuch as it was not essentially different it has not been pictured. Fig. 2 represents a series of graphs made from the corneal cells of 14 urethane-treated and 14 control mice. The stages and designations are the same as for the rats (Fig. 1). It is obvious that response through decline in the number of mitotic figures, and persistence of the inhibition were even more pronounced than in the rat.

It is evident that instead of arresting mitosis in metaphase and holding it there as colchicine does, urethane almost wholly does away with all stages of it for a time. In this respect its effects seem more to resemble those described as following ether, cocaine or ephedrin.

In subsequent experiments with urethane we were surprised to find that it not only affects mitosis but causes pulmonary tumors† in well over half (75 out of 86) of our experimental animals. The effect is evidently the same as that reported by Nettleship and Henshaw[3] for this drug on mice.

Summary. Intraperitoneal injections of urethane (1 cc of a 10% aqueous solution per 100 g of body weight) almost wholly does away with all stages of mitosis for a period of 8 to 12 hours, judging from counts of mitotic figures in sections of the corneal epithelium from rats and mice. In a later experiment, lung tumors of adenomatous type appeared in 75 of 86 animals treated with urethane, but what, if any, connection there is between the interference of this drug with mitosis and its causation of lung tumors is not evident.

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† Report in press, *Cancer Research.*

[3] Nettleship, A., and Henshaw, P. S., *J. Nat. Canc. Inst.*, 1943, **4**, 309.

15681

Persistence of Resistance of Tubercle Bacilli to Streptomycin During Passage Through Guinea Pigs.

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Youmans and associates[1] have reported that tubercle bacilli from 8 of 12 patients treated with streptomycin had a resistance to this drug 500 to 1,000 times as great as that of cultures taken before treatment with streptomycin. We have studied cultures from tuberculous patients before and during the course of treatment with streptomycin and also have observed this phenomenon. In some instances cultures of tubercle bacilli have shown a resistance to more than 10,000 times the concentration of streptomycin compared to those obtained prior to treatment.

Preliminary observations indicate that resistance to streptomycin may persist after repeated subculture and storage in the refrigerator. We present herewith our findings that tubercle bacilli isolated from guinea pigs inoculated with sputum, urine or material obtained on gastric lavage from tuberculous patients who were being treated with streptomycin are as resistant as cultures isolated directly from the same materials.

Methods. Specimens of sputum, urine or gastric washings were cultured in 4 tubes of glycerinated egg yolk agar and also inoculated into 2 guinea pigs. Cultures were observed weekly and when growth occurred one tube was selected for determination of resistance to streptomycin. The spleens of the guinea pigs were cultured on the egg yolk agar when the animals died or when they were killed in 8 to 9 weeks. The tests for resistance were done in a manner modified from that used by Youmans and his associates. Two-fold dilutions of streptomycin* ranging from 2,000 to 0.04 μg per milliliter of medium were made in Vorwald's modification of Proskauer and Beck's medium containing 10% horse plasma. This was dispensed in 5 ml amounts into 150 by 15 mm test tubes. The inoculum was prepared by grinding in a mortar and suspending a portion of the culture in Proskauer and Beck's medium.

The suspension of tubercle bacilli was made to contain approximately 1 mg of bacteria per milliliter as determined by comparison with a standard made by use of the Hopkins tube. Each tube of medium was inoculated with 0.1 ml of suspension or approximately 0.1 mg of tubercle bacilli. We have found that the inoculum may vary within relatively wide limits without any variation being reflected in the results. Duplicate tests of tubercle bacilli with a known resistance to streptomycin have given the same results when 0.2, 0.1, 0.05 or 0.025 mg of inoculum was used. After incubation at 37°C for 14 days the resistance to streptomycin was recorded as the greatest concentration that permitted visible growth. When duplicate tests were made or tests were repeated the results occasionally varied to the next higher or lower concentrations. The apparent large differences as in cases 4 and 12 in Table I are inherent in a 2-fold dilution method.

Results. A comparison of resistance to streptomycin between tubercle bacilli cultured directly from sputum, urine or material removed by gastric lavage and those isolated after passage through guinea pigs is given in Table I. Cultures from untreated tuberculous patients usually are resistant to only 0.08 to 0.62 μg of streptomycin per milliliter of medium and the majority have a resistance of 0.15 μg. Our information is not complete but our present data indicate that resistance of tubercle bacilli from patients being treated with streptomycin is not apparent until treat-

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[1] Youmans, G. P., Williston, Elizabeth H., Feldman, W. H., and Hinshaw, H. C., *Proc. Staff Meet., Mayo Clin.*, 1946, **21**, 126.

* Furnished through the courtesy of Merck and Co., Rahway, N.J.

TABLE I.
Age of Culture, Duration of Guinea Pig Passage and Resistance to Streptomycin of Tubercle Bacilli Isolated from Patients by Direct Culture and by Guinea Pig Inoculation.

Case	Direct culture		Culture from guinea pig*		
	Age of culture, days	Resistance to streptomycin†	Days in guinea pig	Age of culture, days	Resistance to streptomycin†
1‡	102	0.15	42	60	0.15
2‡	161	0.15	67	94	0.15
3	31	100.0	64	42	100.0
4	24	>2000.0	22	55	500.0
5	114	25.0	71	43	25.0
6	181	25.0	67	114	25.0
7	34	>2000.0	68	30	>2000.0
8	114	10.0	63	50	10.0
9	24	>2000.0	61	37	>2000.0
10	74	0.08	93	32	0.31
11	70	>2000.0	58	33	>2000.0
12	98	1500.0	65	33	>2000.0
13	95	25.0	76	135	25.0
14	112	0.15	60	52	0.15

* Inoculated the same days as direct cultures were made with a portion of the same material.

† Greatest concentration of streptomycin allowing growth in micrograms per milliliter of medium.

‡ Untreated patients.

ment has been carried out for several months.

It is evident from the data presented in Table I that cultures of tubercle bacilli from patients receiving streptomycin retain their resistance to the drug even after residence in guinea pigs for 10 weeks or more and subsequent maintenance on glycerinated egg yolk agar for many weeks.

In another experiment each of 14 guinea pigs was inoculated subcutaneously with 0.1 mg of tubercle bacilli found to be resistant to at least 2,000 μg of streptomycin per milliliter of medium. Cultures isolated from 9 of these that died after periods of 61 to 164 days of infection were found still to be resistant to at least 2,000 μg. In all 14 animals there was gross and microscopic evidence of progressive tuberculosis indistinguishable from that seen in a similar group of guinea pigs inoculated with a sensitive strain from the same patient.

15682

Uniformity of Size of Bacteriophage Particles.

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As measured by the sintered glass boundary method of Northrop and Anson, Kalmanson and Bronfenbrenner[1] found the apparent diffusion coefficient of coliphage PC (hereafter called T2K) to be about 1.7×10^{-7} cm^2/sec, corresponding to an equivalent spherical diameter of about 16 mμ. By fractional ultrafiltration, a very small fraction of the phage was obtained which behaved in the same measurement like particles having a diameter of about 4 mμ.

Immunological analysis[2] revealed a remarkably high combining power of the phage for neutralizing antibody, about 4600 molecules of antibody per lytic unit of phage. This may be taken as evidence that the preparations of Kalmanson and Bronfenbrenner,[1] weighing about 10^{-13} mg per lytic unit, consisted of remarkably pure antigen derived from the phage. The weight 10^{-13} mg is equivalent to about 30 particles of 16 mμ diameter, and since the measurement of effective antigenic surface seemed to give independent support for this assumption, it was concluded that the total number of phage particles, each measuring 16 mμ in diameter, was about 30 times higher than that indicated by the lytic titre.[2]

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[1] Kalmanson, G. M., and Bronfenbrenner, J., *J. Gen. Physiol.*, 1939, **23**, 203.

[2] Hershey, A. D., Kalmanson, G. M., and Bronfenbrenner, J., *J. Immunol.*, 1943, **46**, 281.

Northrop,[3] both by diffusion and centrifugal analysis, estimated a diameter of about 10 mμ for a staphylococcal phage in dilute solution (in concentrated solution it behaved like particles 100 mμ in diameter). A few phages, namely S13 and C13, have a similar small size (8 to 20 mμ) by either filtration or centrifugal analysis.[4] Otherwise, and particularly for phages belonging to the serological group (group 11 of Burnet[5]) homologous with T2K, much larger sizes have been found by all workers. For example, phage WLL has a diameter of 50 to 75 mμ by filtration, and 60 mμ by sedimentation.[4] T2K itself has a diameter of about 50 mμ estimated from the ratio of weight to lytic titre of the best preparations,[1] and T2 (a variant of T2K now known as T2L[6]) has a diameter of about 50 mμ from the X-ray sensitive volume,[7] and measures about 65 × 80 mμ[8] or 80 × 100 mμ[9] in electron-micrographs. In addition, approximate one to one correspondence between lytic titre and particle count revealed by means of the electron-microscope,[8] contradicts our conclusion[2] regarding the infectivity of the particles.

In view of this unsatisfactory state of affairs,[4] we began several years ago some additional measurements of the size and uniformity of phage T2K. We have only now been able to correlate our results in a fairly satisfactory way.

We have used 3 methods: diffusion through agar, centrifugation, and diffusion across the sintered glass boundary. In all 3 methods, another coliphage, T1, has been used as reference, the 2 phages being counted separately in mixtures by plating on suitable indicator strains of bacteria each sensitive to only one of the phages.

Diffusion Through Agar. Diffusion through agar was studied as follows. Suitable quantities of melted 3% washed agar in distilled water, and the phages in broth, were mixed at 45°C to give a 0.6% agar solution containing about 10^7 particles each of T1 and T2K per ml. The phages consisted of filtered broth lysates diluted about 1:1000. At the same time a similar solution was prepared containing broth without added phage. The boundary was set up by allowing 10 ml of the phage-free solution to solidify in the lower end of a length of glass tubing (16 mm internal diameter) fitted with rubber stoppers, and then adding an equal volume of the phage-containing solution. After this had solidified, a layer of melted paraffin about 2 cm long was pipetted directly onto the agar surface. This formed a plug serving to minimize mechanical movements of the gel during diffusion, which was allowed to proceed at 5°C in the horizontal direction.

Ultrafiltrates were prepared by passing broth lysates through a collodion membrane of sufficient density to reduce the lytic titre by the factor 10,000.[1] The diffusion measurements were conducted with these fractions in the way already described, except that the ultrafiltrates were incorporated into the agar gel without further dilution, yielding a titer of about 10^5 per ml, and the corresponding phage-free solution contained the same ultrafiltrate inactivated by heating, instead of broth. In these experiments also, unfractionated T1 admixed in equal titer

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[3] Northrop, J. H., *J. Gen. Physiol.*, 1938, **21**, 335.

[4] Elford, W. J., in *Handbuch d. Virusforschung*, Doerr and Hallauer, Editors, Vienna, 1939, p. 126.

[5] Burnet, F. M., *J. Path. and Bact.*, 1933, **36**, 307.

[6] Hershey, A. D., *Genetics*, 1946, **31**, 620.

[7] Luria, S. E., and Exner, F. M., *Proc. Nat. Acad. Sci.*, 1941, **27**, 370.

[8] Luria, S. E., Delbrück, M., and Anderson, T. F., *J. Bact.*, 1943, **46**, 57.

[9] Hook, A. E., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Biol. Chem.*, 1946, **165**, 241.

TABLE I.
Diffusion Coefficients of T1 and T2K in Agar.

Material	Time (days)	Phage per ml	$D_1 \times 10^8$	$D_2 \times 10^8$	D_2/D_1
Whole T1 + Whole T2K	7	*ca.* 10^7	1.6	0.98	0.61
	14		0.82	0.53	0.65
	28		0.61	0.44	0.72
	43		0.69	0.50	0.72
Whole T1 + Ultrafiltered T2K	14	*ca.* 10^5	1.9	1.4	0.74

D_1 = diffusion coefficient of T1.
D_2 = diffusion coefficient of T2K.

served as reference.

For analysis of the contents of the diffusion cell, the agar was cut into segments with a fine stretched wire, beginning from the phage-free end of the column, the latter being expelled from the tube without backward movements by means of the plunger formed by anchoring a wire handle in the paraffin plug. The segments, collected in numbered weighing bottles, were weighed, and their length and position with reference to the boundary fixed by summing the weights. The phage content of the segments was determined by suspending the agar in broth by aspiration with a pipette, and plating after suitable dilution. The recovery of both phages under the conditions of the experiments was quantitative.

Owing to the nature of the analytic method, and to the slow rate of diffusion, only the data for the region of low phage concentration proved useful for the calculation of diffusion coefficients. This disadvantage, as compared with optical methods of analysis in the diffusion of purified proteins for instance, was more than offset by the advantages of being able to use very dilute solutions, and to make analysis from the region of 10^{-1} down to about 10^{-5} times the initial concentration of phage.

For this region, the distribution of phage about the boundary, after correction for the lengths of the individual segments (by graphical integration of the ideal diffusion curve), conformed nearly but not quite to that expected for ideal diffusion. The deviations were systematic, and might be attributed to heterogeneity of the phage, to the curvature of the boundary, or to seepage of fluid along the walls of the tube.

The pertinent results of 2 experiments are summarized in Table I. It will be seen that the method clearly distinguishes T1 and T2K in mixture, the latter diffusing more slowly. The diffusion coefficients, calculated for the largest distance from the boundary at which dependable analysis could be made, vary from 0.61 to 1.9×10^{-8} cm²/sec for T1, and from 0.44 to 1.4×10^{-8} for T2K. The variation must arise either from errors in locating the boundary, or to differences in the quality of the 2 preparations of agar gel, since the relative coefficients of the 2 phages vary only from 0.61 to 0.74. Since this ratio is not altered by ultrafiltration of T2K, we evidently failed to obtain any fractionation with respect to size of particles of this phage by this method.

The actual size of the particles cannot be estimated from these data, owing to the impeding effect of the gel on diffusion. It is of some interest to note the magnitude of this effect. For T2K, the diffusion coefficient of 0.5×10^{-8} corresponds to an equivalent spherical diameter of about 500 mμ, calculated for diffusion in water. This is about 6 times the probable true diameter,[8] as if the particles were diffusing freely in the agar gel only about one-sixth of the time allowed. On the other hand, the relative sizes estimated from these data, namely T1 about two-thirds the diameter of T2K, is in good agreement with the corresponding X-ray sensitive volumes[7] and electron-micrographs.[8]

Other experiments using this same method, in which a second ultrafiltrate of T2K was compared with whole T1, and an ultrafiltrate

TABLE II.
Sedimentation of Fractions of T2K in Mixture with T1.

		% sedimented			
		T1		T2K	
Source of material	No. of Exp.	Mean	Extremes	Mean	Extremes
Whole broth lysates	7	14	4-28	84	61-99
Ultrafiltered T2K + whole T1	6	13	5-34	91	76-99
Supernates, T2K	3			87	69-97

of T1 with whole T2K, yielded less presentable data, but nevertheless confirmed our principal conclusion, that ultrafiltration does not affect the speed of diffusion of these 2 phages relative to each other. The 3 ultrafiltrates examined in this way were prepared with 3 different collodion filters.

Centrifugation. The sedimentation experiments were also essentially qualitative, but yielded unequivocal results owing again to the use of a second phage as standard of reference. The method follows.

Three ml volumes of the mixed phages in broth (about 10^5 lytic units per ml) were spun for one hour at 18,000 r.p.m. in 6 ml tubes in an international centrifuge equipped with "multispeed" angle head. The mean distance from the axis of rotation was about 6.5 cm, corresponding to a centrifugal field of 23,500 g. When spun in a cool room, the temperature in the tubes did not exceed 45°C, and the phage could be recovered quantitatively by mixing the contents of the tube at the end of the run. Supernates were sampled by withdrawing 0.5 ml with a pipet from the upper portion of the fluid. Assays of these samples were compared with the same materials uncentrifuged, and in many of the experiments an assay was made also of the mixed residue in the bottom of the tube, as a parallel check against inactivation. Table II shows the results of several experiments with T1 and T2K from lysates prepared in broth, with ultrafiltrates of T2K prepared with 2 different collodion filters, and with T2K from supernates of previous centrifugations. We have omitted from these data a few discrepant experiments, in which both phages failed to sediment appreciably, since this happened just as frequently with unfractionated as with fractionated phage, and can safely be attributed to accidents of convection.[10] Excluding these, the results show clearly that:

(1) T2K sediments rather completely under the conditions of these experiments, whereas admixed T1 is left largely in the supernate.

(2) The few particles of T2K passed by 3% collodion membranes sediment just as rapidly in the ultrafiltrate as whole phage diluted to the same titer in broth. This conclusion must be qualified somewhat, owing to inaccuracies of the method, but it is clear that we have failed to obtain a fraction of T2K containing particles anywhere near as slowly sedimenting as those of T1.

(3) T2K in supernates from which the bulk of the phage has been removed by previous centrifugation, is not less easily sedimentable than the unfractionated material. In another experiment not shown in the table, 3 successive centrifugations of an undiluted lysate threw down respectively 98.5, 96, and 97% of the residual unsedimented phage.

We conclude that the particles of T2K in dilute solution are essentially homogeneous in size, or at any rate all are appreciably larger or denser than those of T1.

Passage through the sintered glass boundary. In a single experiment, carried out as described by Kalmanson and Bronfenbrenner,[1] it was found that whole T2K in broth, and ultrafiltered T2K from a broth lysate, passed through the sintered glass boundary into broth, or into heated ultrafiltrate, respectively, at an identical rate corresponding to a spherical diameter of about 4 mμ. In both cells, the admixed T1 and T2K passed in precisely the same proportion.

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[10] Stanley, W. M., *J. Exp. Med.*, 1944, **79**, 267.

Since particles of this size would not be sedimentable under the conditions of the centrifugations described above, it is evident that we are not here measuring diffusion, and this conclusion is confirmed by the failure to distinguish T1 and T2K in the same mixture. Probably what we are measuring is actual circulation of fluid through the boundary.

A repetition of the experiments with phage propagated in synthetic medium nevertheless confirmed the earlier results, namely, that whole lysate diluted in synthetic medium passed the boundary at an appreciably slower rate than undiluted ultrafiltered phage, which passed at essentially the same rate as phage in broth. This result is evidently inconsistent with the conclusions reached above.

It was noticed, however, that the synthetic medium used in these experiments[1] was supersaturated with respect to magnesium ammonium phosphate, and eventually deposited crystals on standing in the refrigerator. Since no crystals formed in ultrafiltrates of lysed cultures prepared in the same medium, it was suspected that the difference in the rate of passage through the glass boundary might be due to the clogging of the porous plate by crystal formation, which might be expected to occur preferentially within the pores of the glass. The following experiment seems to show that this is what happens.

Parallel measurements of the rate of passage through the glass boundary were made of the same stock of phage T2K diluted respectively in a "saturated" and a "supersaturated" medium. The supersaturated medium was prepared by mixing the 2 stock solutions[1] composing the medium, and placing the mixture at 5°C for about 12 hours, at which time crystallization had not yet begun. The saturated medium was prepared from the same materials, but was stored in the refrigerator for several days, or as long as necessary, until crystallization was complete, and filtered. Two experiments were done, switching the cells used for the measurement of saturated and supersaturated solutions, and cleaning the cells between runs with chromic-sulfuric acid mixture. The results, expressed as equivalent spherical diameters calculated from the apparent diffusion coefficients, were as follows: in saturated medium, 6.8 and 9.8 mμ, and in supersaturated medium, 22.2 and 24.4 mμ. This difference is clearly an artifact, and very likely has the explanation we have suggested.

Discussion. As a result of the experiments described in this paper, it must be concluded that the unit particles of the phages T1 and T2K in diluted lysates are of very large size. They are probably identical with those identified in undiluted lysates by electron-micrography,[8,9] analytical centrifugation,[9] and other means.[4,7] There is no evidence that these particles are heterogeneous in size, or that they disaggregate appreciably on dilution. The previous conclusion,[2] based partly on immunological evidence, that lysates are composed of antigenically similar particles about 30 times more numerous than indicated by the lytic titer, has also to be withdrawn. The infectivity of the phage particles must be very nearly perfect, as has been found also by other means.[8]

The very large antibody absorbing capacity[2] of bacteriophage T2K, which is equally great for several members of the same serological group,[6] may now be accounted for in terms of the analytical data for T2 reported by Hook and co-workers.[9] These data, which refer to morphologically and centrifugally homogeneous preparations, indicate a considerably larger size than any of the previous estimates. The electron-micrographs show particles 80 × 100 mμ or 86 × 113 mμ, exclusive of tail, depending on the source of material. From the sedimentation constant (which, however, unaccountably decreases at low concentrations) one estimates an equivalent spherical diameter of 60 to 130 mμ for the densities 1.5 to 1.1 respectively. From the weight per lytic unit of 10^{-15} gm, and assuming perfect infectivity, one estimates a spherical diameter between 100 and 150 mμ, depending on the density and hydration assumed. A sphere of diameter of 100 mμ, plus a tail of 20 × 120 mμ,[8] provide a primary physical surface of about 4×10^{-10} cm^2, sufficient to accommodate 4000 molecules of antibody each requiring an area of 10^{-13} cm^2. This size is therefore compatible with the observed combining ratio of 4600 molecules per lytic unit,[2] a correlation which also holds with fair exactness for antigens as diverse in size and shape as ovalbumin, thyroglobulin, hemocyanin,[11] and tobacco mosaic virus.[12]

These experiments have some bearing on the question of the utility of the porous plate boundary method of Northrop and Anson,[13] for the measurement of coefficients of diffusion. In this method, the estimated coefficient is directly proportional to the fraction of the solute diffusing in unit time. Under our conditions, the rate of flow of fluid through the boundary, measured in terms of the amount of bacteriophage passed, is about 0.01 ml per hour, equivalent to a diffusion coefficient of about 4×10^{-7} cm^2/sec. This is of the same order as the diffusion coefficient of hemoglobin.[13] It is evident that this rate of flow would not seriously affect the measurement of diffusion of substances diffusing 5 to 10 times faster than hemoglobin, *e.g.*, of the HCl or other electrolyte used in calibration. For proteins of the size of hemoglobin, the error would be about 100% for solutions having a viscosity near that of water. Actually, protein solutions of sufficient concentration for analysis of the diffusate have a considerably higher viscosity, and the data of Northrop and Anson[13] suggest that under their conditions, the flow of fluid is negligible compared with the diffusion of hemoglobin, which means that the flow is at least several times slower than in our experiments. Bacteriophage T2, taken to be a sphere of about 90 mμ in diameter, would have a diffusion coëfficient of about 2.7×10^{-8} cm^2/sec in water, and would pass through glass discs such as we have used at a rate equivalent to about 6×10^{-4} ml of solution per hour by diffusion alone. Observed amounts in excess of this would provide a very accurate measure of the speed of flow of liquid. A small amount of bacteriophage included in the solution whose diffusion is to be measured would thus permit a very simple correction for what is probably the principal source of error in the use of this method.

Summary. Neither fractional ultrafiltration nor fractional centrifugation have revealed any heterogeneity of size among the particles of the bacteriophage T2K (formerly called PC). The methods used for the measurement of size, namely, diffusion through agar and sedimentation in an angle centrifuge, were essentially qualitative, but the experimental arrangements were such as to yield unequivocal results.

It must be concluded therefore that this bacteriophage has a minimal particle diameter of 60 mμ or more, as has been found previously for unfractionated phages of the same group by a variety of methods.

It is necessary also to withdraw the conclusion previously reached that this bacteriophage has a low infectivity for susceptible bacteria.

An explanation is suggested for some anomalous results previously obtained in attempts to measure the diffusion of bacteriophage through the porous glass boundary. The use of bacteriophage to measure the rate of flow of fluid as a means of control in the application of this method to other materials is described.

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[11] Hershey, A. D., *J. Immunol.*, 1941, **42**, 485.

[12] Schramm, G., and Friederich-Freksa, H., *Z. physiol. Chem.*, 1940, **270**, 233.

[13] Northrop, J. H., and Anson, M. L., *J. Gen. Physiol.*, 1929, **12**, 543.

15683

Effect of 2,3-Dimercaptopropanol (BAL) on Acute Poisoning by Tervalent and Quinquevalent Antimonial Drugs.*

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Among the numerous reports on the experimental use of BAL to combat poisoning by inorganic and organic metallic compounds, the investigation of Braun, Lusky and Calvery[1] is the only one describing work with antimonial drugs. These authors concluded from their work with rabbits that "With the administration of BAL, the tolerance of the rabbits to lethal doses of compounds of antimony was increased more than 50%." Our experiments were all performed with young rats in which we were able to demonstrate a protective action of BAL against only one antimonial, tartar emetic. The lethal action of 4 other antimonial drugs, including Fuadin and Neostam which were also used by Braun, Lusky and Calvery, either was not affected or was increased by the administration of BAL.

Methods. All the experiments were performed with young albino rats (Sherman strain) usually weighing about 75 g (range 55-140 g). A total of 210 female and 885 male rats was used. Each group, usually of 10 animals, if treated with BAL, had a companion group of 10 of the same weight range and sex receiving only the antimonial drug. The dose of antimonial chosen was sufficient to kill 60-100% of animals in the preliminary experiments with single doses. If the BAL had no clearly favorable influence on the lethal action of the drug, the dose of the latter was reduced to determine whether there was an appreciable beneficial or deleterious action of BAL. In some experiments, smaller doses of an antimonial drug and of BAL were administered repeatedly.

In every experiment the antimonial and the BAL solutions were administered by different routes. When an aqueous solution of the antimonial drug was injected intraperitoneally, BAL in oil, according to the formula of Eagle,[2] was given subcutaneously after dilution of the solution by peanut oil. Solutions of BAL in oil were unsatisfactory for repeated injection over a period of days owing to the accumulation of unabsorbed oil. In later experiments aqueous solutions of drug and of BAL were injected by the same routes as those used by Stocken and Thompson[3] in experiments with sodium arsenite; the antimonial drug was injected intramuscularly and freshly-prepared aqueous BAL solution was given intraperitoneally. The antimonial drugs tested were tartar emetic (antimony potassium tartrate). Fuadin[†] (NaSb-bis-pyrocatechindisulfonate of Na), Neostibosan[†] (Sb complex of *p*-aminophenylstibonic acid, *p*-acetylaminophenylstibonic acid, antimonic acid and diethylamine), Neostam[‡] (nitrogen-glucoside of sodium *p*-aminophenylstibonate), and Stibanose[†] (Solustibosan or monohydroxytriethylammonium salt of 2,3,antimonic ester of sodium hexonate). The antimony of tartar emetic and of Fuadin is tervalent, whereas it is quinquevalent in the other 3 compounds. Stibanose is of such low toxicity that the large volume of concentrated solution required for a lethal effect could not be injected intramuscularly. The aqueous solutions of Neostam and Neostibosan for intramuscular administration contained respectively 20 g and 30 g of drug per 100 ml of solution.

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* This investigation was aided by a grant from the Sterling-Winthrop Research Institute.

[1] Braun, H. A., Lusky, L. M., and Calvery, H. O., *J. Pharm. and Exp. Therap., Suppl.*, 1946, **87**, 119.

[2] Eagle, H., *J. Ven. Dis. Inf.*, 1946, **27**, 114.

[3] Stocken, L. A., and Thompson, R. H. S., *Biochem. J.*, 1946, **40**, 535.

[†] Kindly supplied by the Sterling-Winthrop Research Institute.

[‡] Kindly supplied by the Burroughs-Wellcome Company.

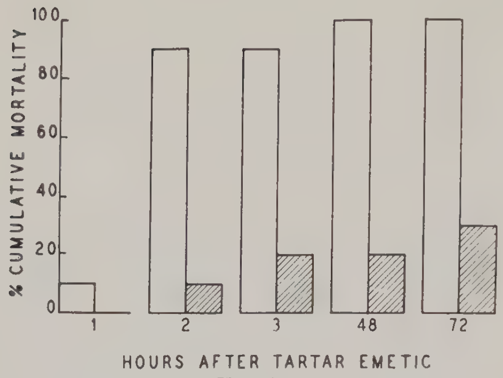

Fig. 1.

Effect of BAL-treatment in the rat on the lethal action of one intramuscular injection of 60 mg of tartar emetic per kg. A saturated aqueous solution of BAL (about 6%) was injected intraperitoneally 5 minutes after tartar emetic in a dose of 0.1 ml per 100 g (about 60 mg per kg). Half of this dose of BAL was repeated 3, 6, and 24 hours after the tartar emetic. One drug-control rat died between the 3rd and the 11th hours of the experiment; no BAL-treated rats died after 72 hours. Each group consisted of 10 rats.

White columns: drug alone.

Shaded columns: drug followed by BAL-treatment.

Results. Ten experiments were performed with tartar emetic administered either intraperitoneally or intramuscularly. In every experiment except one, treatment with BAL either increased the survival period or prevented death, in comparison with groups of rats simultaneously receiving only the solution of tartar emetic. Illustrative experiments are shown in Fig. 1 and 2. A control experiment with very large doses of $HgCl_2$ as the lethal agent is shown in Fig. 3; an expected protective action of BAL was demonstrated although more survivals after the last dose probably would have been observed had BAL treatment been continued after the last injection of $HgCl_2$ solution. In the one inconsistent experiment, tartar emetic in increasing doses was injected once daily intraperitoneally into each rat of 3 groups of 10 each. The initial dose was 30 mg per kg and the last dose, on the 11th day, was 60 mg per kg. However, only 20% of the control rats died whereas of those receiving BAL in oil in addition to tartar emetic, 80% of the second group (20 mg of BAL per kg immediately after tartar emetic) and 30% of the third group (20 mg of BAL per kg immediately after tartar emetic and repeated 3 hours later) did not survive. The subcutaneous tissues of the animals treated with BAL contained several milliliters of oil at the end of the experiment. No satisfactory explanation of the deleterious effect of BAL-treatment or of the remarkable tolerance of the control rats to intraperitoneally-administered tartar emetic in this experiment has been found.

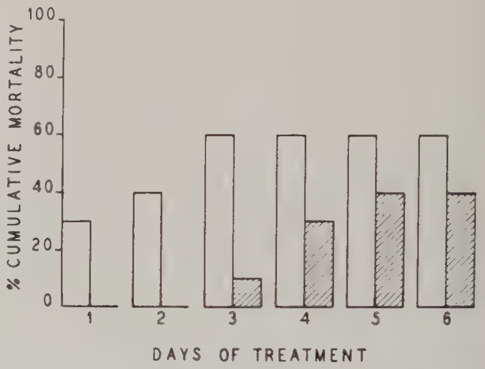

Fig. 2.

Effect of BAL in oil subcutaneously on survival after intraperitoneal injection once daily of 25 mg of tartar emetic per kg in aqueous solution. One dose of BAL (20 mg per kg) was given immediately after each of the 6 injections of tartar emetic. There were no deaths after the 6th injection. Each group consisted of 10 rats.

White Columns: drug alone.

Shaded columns: drug followed by BAL-treatment.

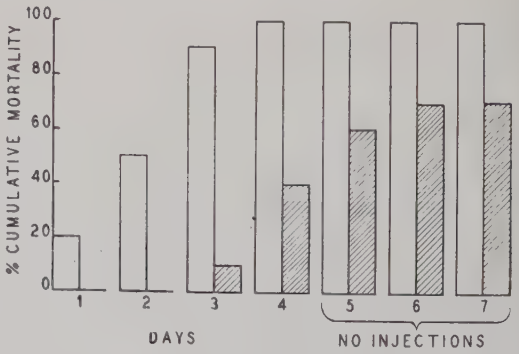

Fig. 3.

Effect of BAL in oil subcutaneously on survival after intraperitoneal injection of 5 mg of $HgCl_2$ per kg once daily at beginning of each of first 4 days. One dose of BAL (20 mg per kg) was given immediately after each injection of $HgCl_2$ solution. The 3 surviving rats were sacrificed. Each group consisted of 10 rats.

White columns: $HgCl_2$ alone.

Shaded columns: $HgCl_2$ and BAL.

Six experiments were performed with the other tervalent antimonial, Fuadin. In one experiment with BAL in oil the mortality in the drug control group was identical with that in the BAL-treated group. In all the other experiments, whether aqueous BAL or BAL in oil was administered, BAL shortened greatly the period of survival and the rate of recovery from toxic or LD_{40-60} doses of Fuadin. This is clearly shown in the experiment of Fig. 4A. A control experiment demonstrating the efficacy of aqueous BAL in arsenite poisoning is illustrated in Fig. 4B, confirming the report of Stocken and Thompson.[3]

Two experiments with aqueous solutions of Neostam and BAL demonstrated that treatment with BAL causes an earlier death (at a dosage of 600 mg of Neostam per kg) or increases the mortality rate (at a dosage of 450 mg of Neostam per kg). The latter experiment is summarized graphically in Fig. 5. Neostibosan was the quinquevalent antimonial used in 6 satisfactory experiments in which single or repeated daily injections were made. Subcutaneous BAL in oil or intraperitoneal aqueous BAL increased the mortality rate. For example, after 1500 mg of Neostibosan per kg intramuscularly, 50% of the BAL-treated animals and none of the drug controls were dead after 3 hours; at the end of the experiment, 40% of the drug control group were dead and 70% of the group receiving intraperitoneal BAL as well as Neostibosan had succumbed. The low toxicity of Stibanose required intraperitoneal injections (*e.g.*, a single dose of 7000 mg per kg caused only 20% mortality) and the BAL was administered in oil subcutaneously. The effect of BAL (20 mg per kg as a single dose or 2 doses 4 hours apart) apparently was to increase the mortality rate, but this effect was not shown to be significant.

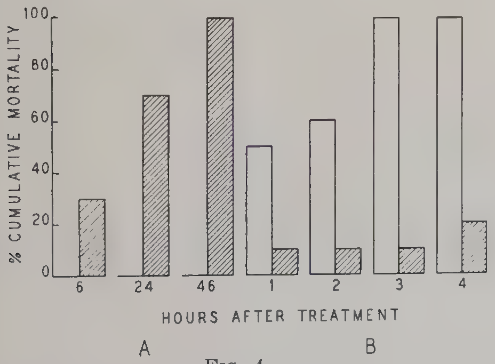

FIG. 4.

A. Lethal effect of treatment with BAL in rats receiving a single intramuscular dose of 250 mg of Fuadin per kg. Without BAL-treatment no rats died within 96 hours. In the group of rats receiving 0.1 ml of saturated aqueous solution of BAL per 100 g (about 60 mg per kg), 70% died within 24 hours and all were dead within 46 hours. Each group consisted of 10 rats.

B. Protective action of BAL in arsenite poisoning. Each rat received, per kilogram, an intramuscular injection of 18 mg of As_2O_3 neutralized by NaOH. The intraperitoneal injection of saturated aqueous solution of BAL was given 10 minutes after the arsenite (0.1 ml per 100 g); a second dose of BAL (0.05 ml per 100 g) was injected 4 hours later. There were no deaths after 4 hours. Each group consisted of 10 rats.

White columns: arsenite alone.

Shaded columns: Fuadin and BAL (A) or arsenite and BAL (B).

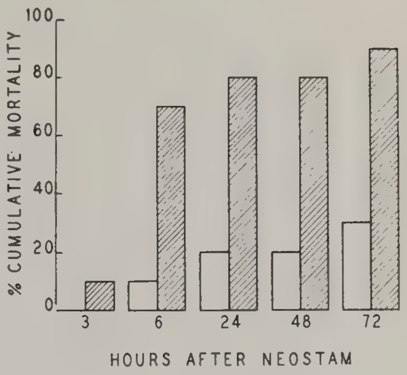

FIG. 5.

Lethal action of BAL in rats receiving a single intramuscular injection of 450 mg of Neostam per kg. BAL, as a single intraperitoneal injection of 0.1 ml of a saturated aqueous solution per 100 g (about 60 mg per kg), was given 5 minutes after the injection of Neostam. No further deaths occurred between the 72nd and the 240th hours. Each group consisted of 10 rats.

White columns: Neostam alone.

Shaded columns: Neostam and BAL.

Discussion. The findings here reported, in which rats were the experimental subjects, demonstrate that BAL is of real value in lessening the mortality rate of animals receiving single or repeated doses of tartar emetic. This conclusion is in agreement with that reached by Braun, Lusky and Calvery, whose experiments were all performed with rabbits receiving intramuscular injections of aqueous solutions of drug or of drug and of BAL. The lethal effects of the other 2 antimonials used by these authors, Fuadin and Neostam, contrary to their results, were found clearly to be enhanced by the administration of BAL. Similarly, BAL increased the lethal action of poisonous doses of Neostibosan. The dimercaptan did not alter the death rate from large intraperitoneal doses of Stibanose. The doses of BAL, whether dissolved in peanut oil or in water, were sufficient to save the lives of animals receiving lethal doses of either mercuric chloride or sodium arsenite. BAL in oil was administered in doses well below levels which by themselves are followed by any manifestation of poisoning. The largest intraperitoneal dose of aqueous solution of BAL used (about 60 mg per kg) can cause central stimulation in some rats, as shown by generalized muscular hyperactivity; however, this dose, followed by 30 mg per kg 4 hours later, can be given daily for at least 3 days without killing any animals.

Other experiments will have to be performed to determine, if possible, what is the explanation of the increased toxicity of Fuadin, Neostam and Neostibosan, if BAL be administered a short time later. The increased mortality associated with BAL-treatment occurred mainly within 24 hours; a significant difference in mortality was evident after 6 hours. It is possible that an acutely toxic complex substance is formed *in vivo* by the interaction of the dimercaptan and any of the 3 antimonials which themselves are not well-defined compounds; however, it is more acceptable to interpret the increased mortality as a summation of toxic effects. It seems reasonable to conclude that the favorable action of BAL in rats acutely poisoned by tartar emetic is the result of the formation of one or more mercaptides of antimony much less toxic than tartar emetic, just as is the case when an arsenical or a mercuric salt is rendered innocuous by the administration of BAL.

Summary. In rats, the acute lethal action of tartar emetic is significantly reduced by the administration of BAL. BAL increases the mortality rate in rats receiving Fuadin, Neostam or Neostibosan. The mortality rate in rats given large doses of Stibanose is not reduced by BAL.

15684 P

Electron Micrographs of Bacterial Cultures Infected with Bacteriophage.

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Now that we have learned to recognize individual virus particles through the electron microscopy of purified suspensions it has become practical to approach the more important question of how these particles are produced within the cells they infect. Probably bacteria with their bacteriophage provide the simplest virus system immediately accessible to existing technics. This is partly because sperm-like bacteriophage particles[1,2] can easily be recognized even in the presence of other particles of similar submicroscopic size and partly because bacteria are hosts small enough to allow an electron microscopic examination of both their surface and their internal structures.

To gain a satisfactory understanding of the steps involved in bacteriophage-production it is essential to be able to photograph sensitive bacteria under the conditions in which they grow and become infected. We have found that this can be done by studying replicas made of the surfaces of agar

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[1] Luria, S. E., Delbrück, M., and Anderson, T. F., *J. Bact.*, 1943, **46**, 57.

[2] Sharp, D. G., Taylor, A. R., Hook, A. E., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 259.

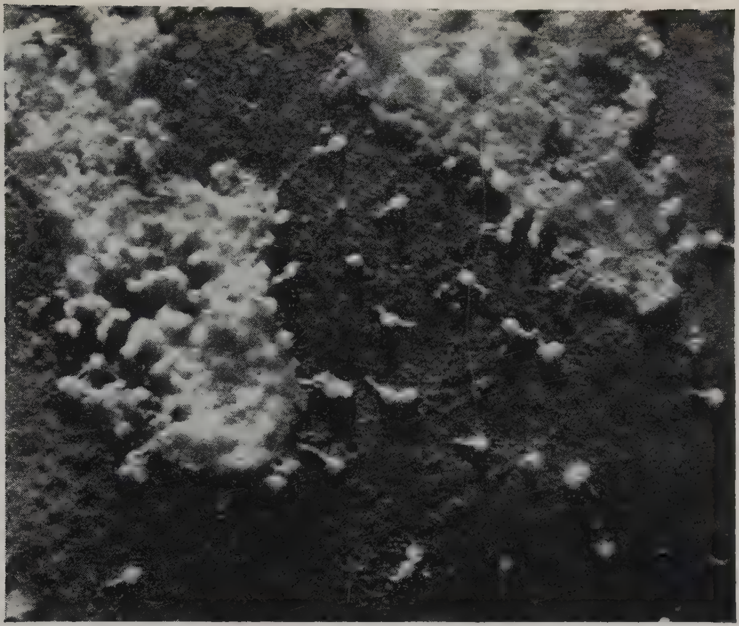

FIG. 1.

An electron micrograph of a gold-shadowed collodion replica of the surface of an *E. coli*-bacteriophage culture. The clear area of the plaque, besides showing a few adhering bacteriophage particles, is covered with pits which presumably are replicas of the heads of other particles. The finer granulation of this and the succeeding photographs is the macromolecular texture of collodion as brought out by the shadowing. Magnification *ca.* 19000 ×.

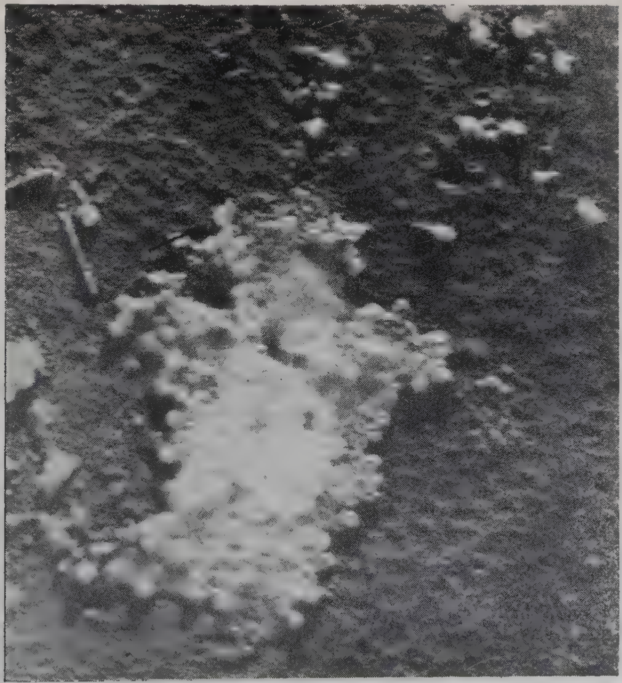

FIG. 2.

A clump of bacteriophage particles just inside the edge of a plaque area on a Petri dish culture. Magnification *ca.* 24,000 ×.

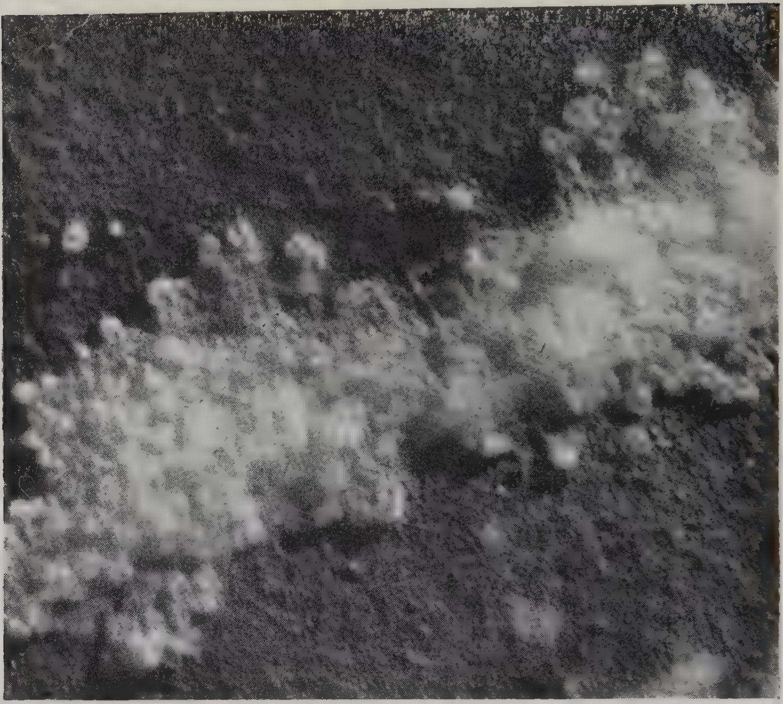

FIG. 3.
Clumps of bacteriophage filling the approximate outlines of two recently divided bacteria. Magnification *ca.* 35,000 ×.

plates incubated for various periods of time after inoculation with bacteria and bacteriophage. The technic we have been using to obtain such replicas is essentially the same as that recently described by Hillier and Baker.[3] It has consisted in covering the surface of a bacteriophage-bacterial growth with a dilute solution of collodion or other plastic and floating the resulting film off onto a water surface for further manipulation. Like Hillier and Baker we have found that organisms are retained by the film made in this way but our preparations have also shown numerous impressions of individual bacteria. This has made it possible to see in a single film details of both surface and internal structure.

Formvar dissolved in ethylene dichloride will yield good replicas but best results have been obtained with collodion. Collodion, USP, diluted with 5 to 6 volumes of normal amyl acetate, carefully spread over the growth-surface and drained till dry has produced films of the proper thickness. The degree of moistness of the surface has had a dominant influence on both the quality and the usefulness of the plastic film. When it was too wet the replica has reproduced little besides micro-drops of water covering its surface; when it was too dry masses of bacteria too thick for observation adhered to the finished replica. Contrast for microscopy has been introduced into the replicas floated from these Petri dishes by shadowing[4] them obliquely with metal to a calculated thickness of ca 8A of gold or ca 40A of chromium. Such shadowed replicas have been so thin that they have often stretched and distorted under the electron beam especially in and about regions of bacterial replication. This could be minimized by lightly metallizing the finished shadowed replicas through the vertical deposition of a few angstroms' thickness of aluminum, chromium or beryllium.

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[3] Hillier, J., and Baker, R. F., *J. Bact.*, 1946, **52**, 411.

[4] Williams, R. C., and Wyckoff, R. W. G., *J. Applied Physics*, 1946, **17**, 23.

Typical micrographs of replicas made in the way just described are shown in Fig. 1-3. They are from the periphery of plaques developed on Petri dishes inoculated with a mixture of *E. coli* and a T2 strain of its bacteriophage. A number of isolated bacteriophage particles adhering to the film are evident in the clear areas of Fig. 1; the film also shows numerous pits that are undoubtedly replicas of particles that remained on the agar. The mass at the left of this micrograph, which approximately outlines the remains of a single bacterial cell, is a typical example of how completely bacterial protoplasm becomes converted into bacteriophage particles after invasion by one of them. Similar masses within the confines of old cells are reproduced at higher magnifications in Fig. 2 and 3. The separate particles can readily be recognized even when, as is commonly the case, they have partially collapsed presumably as a consequence of drying. These pictures do not reveal in detail how the bacteriophage particles are formed but they do suggest that a careful study of films taken from cultures containing bacteria in various stages of infection may be expected to yield such information.

15685

Sugar Alcohols. XXV. Sorbitol and Sorbitan as Precursors of Liver-Glycogen in the Rat.*

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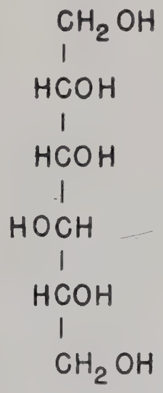
From the University of Maryland, Baltimore, Md.

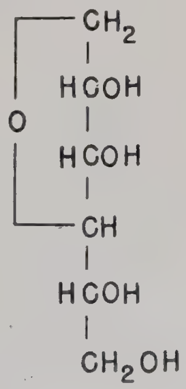
For many years we have been concerned with the fate of the sugar alcohols in the animal body.[1] Previously it was shown that mannitol served as a precursor of glycogen in the liver of the rat, whereas its anhydrides mannitan and isomannide were not available in this capacity. Sorbitol, an isomer of mannitol, surpasses the latter as a glycogen former. Having available crystalline sorbitan, anhydrosorbitol, it occurred to us to study its availability as a source of liver glycogen. Previously Carr and Krantz showed that polygalitol-1,5-anhydro-D-sorbitol was not available as a precursor of glycogen in the rat.[2] The relation of sorbitol to sorbitan is shown by the following formulas.

Experimental. Young white male rats from a uniform source, weighing from 150 to 200 g were fed a balanced ration (Purina Chow) for 3 days. They were then fasted for 48 hours and divided into the following experimental groups according to diet.

1. Cacao butter controls.
2. Cacao butter plus 30% crystalline sorbitol.



D-SORBITOL
M.p.97° [α]D -1.97°



D-SORBITAN
Probable structure
M.p. 113°

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* The sorbitol and sorbitan were generously supplied by the Atlas Powder Company of Wilmington, Del.

[1] Carr, C. J., and Krantz, J. C., Jr., *Advances in Carbohydrate Chemistry*, Vol. I, p. 175-192.

[2] Carr, C. J., and Krantz, J. C., Jr., *J. Biol. Chem.*, 1934, **107**, 371.

TABLE I.
Glycogen Storage in Liver of White Rat After Feeding Sorbitol and Sorbitan in a Cacao Butter Diet.

Cacao butter		Sorbitol		Sorbitan	
No. of rats	Liver glycogen, %	No. of rats	Liver glycogen, %	No. of rats	Liver glycogen, %
3	Less than 0.1	3	0.85	3	0.05
3		8	1.16	3	0.02
3		5	1.37	3	0.04
3				3	0.04
3				3	0.05
				3	<0.01
				3	<0.01

3. Cacao butter plus 30% crystalline sorbitan.

Several animals in each group were divided into separate cages containing 3 to 8 rats each. The animals were fed their respective diets for 2 days. At the end of the feeding period the animals were anesthetized with intraperitoneal injections of amytal sodium and their livers extirpated. The livers in each group were pooled. The glycogen was determined by the method of Good *et al.*,[3] and the dextrose obtained by its hydrolysis was determined by the Munson-Walker method. The results are shown in Table I.

Conclusion. The data in Table I show that sorbitol and sorbitan behave like their isomers mannitol and mannitan[4] with respect to glycogen storage in the liver of the white rat. Mannitol and sorbitol are precursors of glycogen whereas their anhydrides are not.

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[3] Good, C. A., *et al.*, *J. Biol. Chem.*, 1933, **100**, 485.

[4] Carr, C. J., *et al.*, *J. Biol. Chem.*, 1933, **102**, 721.

15686 P

Alteration of the Distribution and Excretion of Streptomycin.*

WILLIAM E. NELSON, JOSEPH FORGACS, AND JOSEPH L. KUCERA.
(Introduced by Edwin H. Lennette.)
From Camp Detrick, Frederick, Md.

Phagocytosis of colloidal particles by the reticulo-endothelium and the localization of such particles in inflamed areas under certain conditions are well known phenomena. These processes apparently depend upon several factors, among which are (a) charge of the particle, (b) particle size, and (c) capillary permeability.

It has been observed that penicillin[1] and streptomycin,[2] while effective in eliminating the septicemic phase of certain diseases, fail to provide a complete cure. In the light of previously reported evidence,[3] indicating that liver and spleen levels of streptomycin remain negative while blood levels are within a therapeutic range, it might reasonably be supposed that therapeutic failure[4] is due to

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* Streptomycin used in these experiments was streptomycin hydrochloride.

[1] Robinson, H. J., Thesis, Rutgers University, New Brunswick, N.J., 1943, 43.

[2] Keefer, C. S., Blake, F. G., Lockwood, J. S., Long, P. H., Marshall, E. K., and Wood, B., Jr., *J. A. M. A.*, 1946, **132**, 9.

[3] Kornegay, G. B., Forgacs, J., and Henley, T. F., *J. Lab. and Clin. Med.*, 1946, **31**, 523.

[4] Kelly, E. G., and Henley, T. F., personal communication.

TABLE I.
Average Concentration* of Streptomycin (Units/g of Tissue) in Blood, Liver, and Spleen of Mice.

Hr after injection	Streptomycin-Trypan Blue† Blood	Streptomycin-Trypan Blue† Liver	Streptomycin-Trypan Blue† Spleen	Streptomycin† Blood	Streptomycin† Liver	Streptomycin† Spleen
1	12.0	12.8	59.4	4.0	5.1	0
4	2.5	2.8	6.1	0	0	0
8	0.9	4.2	4.0	0	0	0
25	0.6	6.0	4.9	0	0	0

Concentration of streptomycin in animals which received

* The micro technics of streptomycin assay of Forgacs and Kucera (*J. Lab. and Clin. Med.*, in press) were used in this experiment.

† Each of the 2 large groups contained 20 animals; 5 animals in each group were sacrificed for examination at the post-injection intervals shown.

the inability of the antibiotic to reach intracellularly contained etiologic agents.[5] To test this supposition, intracellular introduction of streptomycin into the reticulo-endothelial cells was attempted by combining the antibiotic with an electronegatively-charged colloid that could be phagocytized. Preliminary experiments consisted of injecting white Swiss mice intraperitoneally with a single dose of a mixture containing 1000 "S" units of streptomycin and 20 mg of an electronegatively-charged dye, trypan blue, in 1 ml of water. The mixture was adjusted to pH 7.4. (In contrast to streptomycin *per se*, this mixture of antibiotic and colloid did not dialyze through a cellophane membrane over a period of 24 hours).[†] Three control groups of animals received respectively (a) 1000 "S" units of the antibiotic dissolved in 1 ml of distilled water, (b) 20 mg of the colloid suspended in 1 ml distilled water, and (c) 1 ml of distilled water. Blood and organs rich in reticulo-endothelial tissue were assayed for the presence of the antibiotic one, 4, 8, and 25 hours after the injection. The average results are presented in Table I.

It will be observed that the group receiving streptomycin in distilled water showed levels of the antibiotic in the blood and liver only in the one-hour samples. The group receiving the antibiotic and dye mixture showed relatively high levels of streptomycin in blood, liver and spleen at all intervals of testing from one through 25 hours. In every case the unitage per gram of liver or spleen in this group always exceeded that of the comparable blood sample. Since the other 2 control groups remained negative throughout the experiment they are not included in Table I. Because of the substantially higher unitages in the liver and spleen of the group which received the antibiotic and dye as opposed to levels in samples of blood taken at comparable times, it was considered reasonable to assume from these data that the antibiotic was concentrated in these organs. Furthermore, histological examination of the sections showed a characteristic distribution of the colloidal dye in the reticulo-endothelium. Results obtained using guinea pigs confirmed the phenomenon observed in mice.

From the above data, it was considered that such a method of altering the distribution of therapeutic substances and delaying their excretion, might be developed for treatment of certain diseases caused by obligately- or facultatively-intracellular parasites.[5] To this end, further experiments are being conducted and will be reported at a later date.

The authors wish to acknowledge their gratitude to Margery T. Fell, Lois L. Chaney, and James W. Brown for their technical assistance, and to Dr. William J. Cromartie for his suggestions and advice.

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[5] Goodpasture, E. W., *Trans. and Studies of the College of Physicians of Philadelphia*, 1941, **9**, 11.

[†] Grateful acknowledgment is made to Dr. Walter Kocholaty for his suggestions and help concerning this phase of the work.

15687

The Use of the Mouse in Studies on Streptomycin.

Geoffrey Rake and Richard Donovick.
From the Division of Microbiology, The Squibb Institute for Medical Research, New Brunswick, N.J.

The therapeutic value of a drug is determined by 2 general aspects; namely, (a) its physiologic distribution in the host and (b) its specific biologic activity. The physiologic distribution may vary greatly with the host as has been pointed out by Marshall[1] with respect to the sulfonamides. The situation with regard to streptomycin is not clear because of widely divergent results which have been published by various investigators.

The mouse, being extensively used in chemotherapeutic studies, is of particular importance in the study of antibiotic substances where the quantities of drug available for research may be sharply limited. Consequently, knowledge of the comparative behavior of streptomycin in mouse and man is of considerable value. The wide range of results which have been reported in the numerous absorption-excretion studies performed in man is summarized in Table I. Aside from an expected variation in individuals, and probably in the assay method and technics, the explanation for the divergence is not clear.

Absorption of streptomycin in mice, as indicated by blood level studies, has been reported[2,9] but no information has been made available on excretion rates. Such data are essential if one is to examine whether streptomycin is destroyed in the mouse to a greater extent than in man.[2] During the past year and a half we have carried out a number of absorption-excretion studies of streptomycin in the mouse as well as in humans (adults and children). Since our findings have bearing on the question of the relative rates of excretion of this antibiotic in these 2 species it was thought to be of interest to present some of these results.

ABSORPTION OF STREPTOMYCIN IN MICE

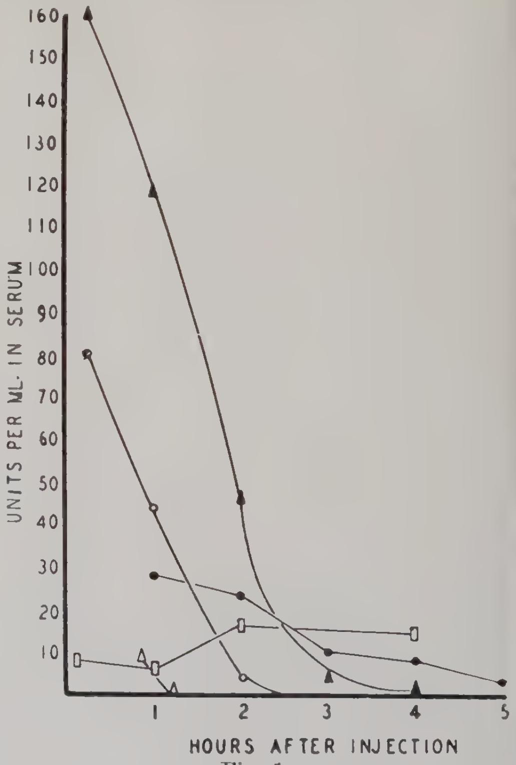

Fig. 1.

▲ 250,000 units per kg. subcutaneously.
○ 104,000 ,, ,, ,, ,,
△ 23,000 ,, ,, ,, intramuscularly.
● 1,140 ,, ,, ,, ,, in children.
□ 500,000 units, intramuscularly in man, cf Buggs, *et al.*[8]

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[1] Marshall, E. K., Jr., *University of Pennsylvania Bicentennial Conference, Chemotherapy,* University of Pennsylvania Press, 1941.

[2] Kornegay, G. B., Forgacs, J., and Henley, T. F., *J. Lab. and Clin. Med.,* 1946, **31**, 523.

[3] Elias, W. F., and Durso, J., *Science,* 1945, **101**, 589.

[4] Heilman, D. H., Heilman, F. R., Hinshaw, H. C., Nichols, D. R., and Herrell, W. E., *Am. J. Med. Sci.,* 1945, **210**, 576.

[5] Rutstein, D. D., Stebbins, R. B., Cathcart, R. T., and Harvey, R. M., *J. Clin. Invest.,* 1945, **24**, 898.

[6] Anderson, D. G., and Jewell, M., *New England J. Med.,* 1946, **233**, 485.

[7] Zintel, H. A., Flippin, H. F., Nichols, A. C., Whiley, M. M., and Rhoads, J. E., *Am. J. Med. Sci.,* 1945, **210**, 421.

[8] Buggs, C. W., Pilling, M. A., Bronstein, B., and Hirshfeld, J. W., *J. Clin. Invest.,* 1946, **25**, 94.

[9] Stebbins, R. B., Graessle, O. E., and Robinson, H. J., Proc. Soc. Exp. Biol. and Med., 1945, **60**, 68.

TABLE I.

Composite Table of Excretion of Streptomycin in Man, Dog, and Monkey as Reported in the Literature.

	Streptomycin administered			
Animal	Route	Dose × 1000 (units)	% recovered in urine in 24 hr or longer	Reference
Man	I.M.	25	6.1-6.5	(8)
	”	50	19.5	(8)
	”	100	59.1-68.1	(4)
	”	200	42-78.2	(8)
	”	200	63 (4 hr)	(6)
	”	300	72	(6)
	”	400	16.7-22.2	(4)
	”	400	41-53.8	(8)
	”	500	56-71	(8)
	”	600	45.5-75.5	(5)
	”	600	87 (12 hr)	(6)
	”	20,000 in 6 days	44	(3)
	”	varied, over 29 days	30†	(2)*
	”	varied, over 25 days	103‡	(2)*
	I.V.	50	50-85.6	(8)
	”	100	52.3-80.0	(4)
	”	200	64-91.7	(8)
	”	400	62.9	(4)
	”	400	42.7-48	(8)
	”	500	79-83.5	(8)
	”	600	29-89§	(7)
	”	28,000 in 7 days	70	(3)
Monkey	I.M.	10 per kg/day	60-66	(9)
	”	50 per kg/day	38-44	(9)
Dog	I.M.	Not indicated	*ca.* 50-80‖	(9)

* Calculated from the published data.

† Total recovery over 29-day period of treatment.

‡ Total recovery over 25-day period of treatment.

§ Average recovery of streptomycin in the urine in 10 cases was 66%.

‖ Recoveries published as approximate figures.

Absorption and Excretion of Streptomycin in Mice. Procedure. Large groups (usually 100) of female Swiss mice weighing between 19 and 21 g were given a single dose of streptomycin subcutaneously or intramuscularly. At given intervals, beginning 15 to 30 minutes after injection, 10 mice were sacrificed by bleeding from the heart, the blood pooled, and assays run on the serum. Immediately prior to each bleeding period, urine samples were taken from all surviving mice (by applying slight pressure over the bladder region) and the urine pooled, measured and assayed by the broth dilution method.[10]*

Results. In Fig. 1 are shown the serum levels reached in mice following a single injection of streptomycin at 3 dose levels. Also included, for purposes of comparison, are a composite curve of similar data we have obtained in infants as well as a curve showing data taken from the work of Buggs, *et al.*[8] In the latter case, the body weight of the patient was not indicated and hence the dosage cannot be given in terms of units per kg. It will be noted that serum levels of streptomycin in mice drop considerably faster than in humans following a single dose of the antibiotic.

The excretion rates as shown by the appearance of antibiotic in the urine of mice as compared to data in humans reported by

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[10] Donovick, R., Hamre, D., Kavanagh, F., and Rake, G., *J. Bact.*, 1945, **50**, 623.

* The various sera studied caused no apparent inhibition of the test organisms. However, assays of sera to which small amounts of streptomycin were added gave results ranging from 34-40% higher than aqueous controls. The streptomycin concentrations were such in these assays that they were carried out on undiluted or low dilutions of serum. Similar studies with low dilutions of urine gave results of 9-23% higher than controls. However, in the absorption-excretion studies in mice the streptomycin concentrations in most of the serum and urine samples were great enough to require considerable dilution of the sample for assay, thereby minimizing this effect to an extent that it may be neglected in the calculations.

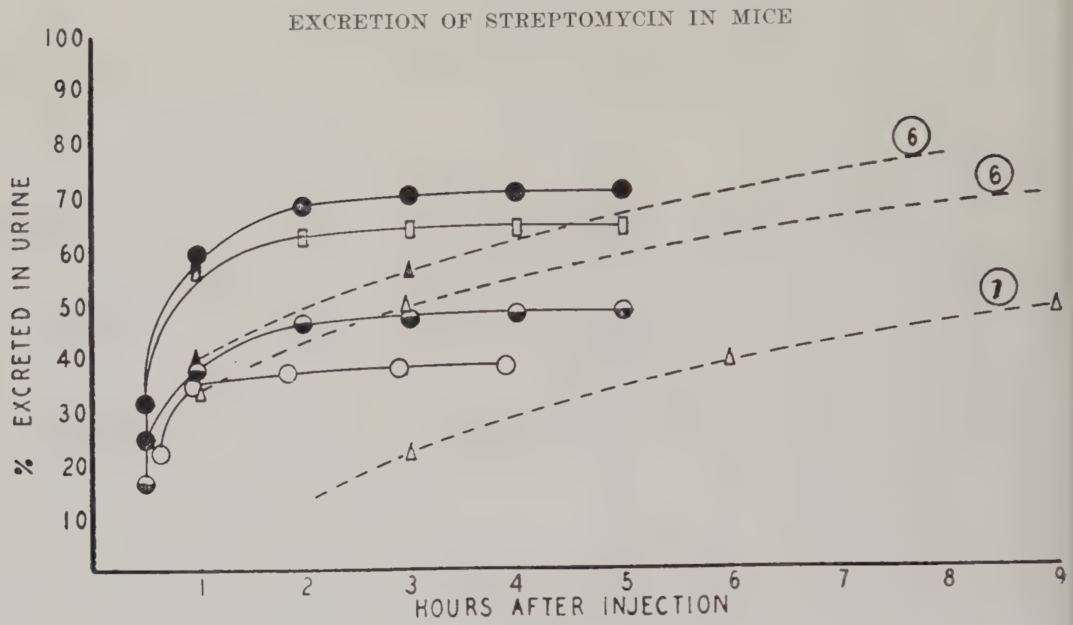

Fig. 2.

● 105,000 units per kg (with alum), subcutaneously.
□ 250,000 ” ” ”, subcutaneously.
○ (half solid) 104,000 units per kg, subcutaneously.
○ 23,000 units per kg, intramuscularly.
▲ 600,000 units, I.M., (total dose) in man, cf Anderson, *et al.*[6]
△ 300,000 ” ” (total dose) in man, cf Anderson, *et al.*[6]
△ 600,000 ” ; I.V., (total dose) in man, cf Zintel, *et al.*[7]

Anderson, *et al.*[6] and Zintel, *et al.*[7] are shown in Fig. 2. Here again, unfortunately, the dosage in humans was not reported with regard to body weight. Nevertheless, it appears evident that the excretion of streptomycin into the urine is more rapid in mice than in man. These data parallel the findings on the rate of decrease in serum levels in these 2 species.

The percentage of streptomycin recovered in the urine of mice by the procedure used must be considered minimal since some urine might have been lost between samples. However, the total recovery was similar to that found in children (see below) and that reported in adults (Table I). Thus, the recovery of streptomycin in the urine ranged from 37.5 to 69.5% of the dose in mice receiving from 460 to 5,000 units intramuscularly or subcutaneously (23,000 to 250,000 units per kg). Except for a more rapid rate of excretion, the mouse does not appear to handle streptomycin differently than does man.

Judging from the data published by Stebbins, Graessle and Robinson[9] the rate at which the blood levels drop in dogs, following a single intramuscular injection, is not greatly different from that in mice. In monkeys it appears to be somewhat slower.

In Fig. 2 it will be noted that one excretion study in mice was carried out with a streptomycin-alum mixture. Carter, *et al.*[11] reported that streptomycin was adsorbed on alkaline alumina. It was therefore of interest to know whether streptomycin injected in mixture with alum was excreted at a slower rate than was streptomycin alone. For this purpose a solution containing 9200 units of streptomycin-sulfate per cc was mixed with an equal volume of 4% aluminum-potassium sulfate and sufficient N/1 NaOH was added to bring to pH 7.6. Female mice were injected subcutaneously with 0.5 cc of the mixture and the urine collected and pooled at given intervals as in the other experiments. In this instance blood levels were not studied.

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[11] Carter, H. E., Clark, R. K., Jr., Dickman, S. R., Loo, Y. H., Skell, P. S., and Strong, W. A., *J. Biol. Chem.*, 1945, **160**, 337.

It was found that the excretion rate of the streptomycin administered together with alum was similar to that of streptomycin alone. The calculated recovery of the antibiotic in the urine was somewhat higher but not significantly so. Apparently the alum did not retard excretion. However, in all previous excretion studies in mice, the urine collected during the first hour or 2 following injection was a deep, reddish brown in color, very similar to that of the concentrated streptomycin solutions used. In the alum study, on the other hand, the urine was normal in color throughout the experiment, indicating that some of the pigmented impurities of the streptomycin preparation had been retained by the alum while the streptomycin was not.

Excretion of Streptomycin in Humans. Considerable data on the absorption and excretion of streptomycin in humans has already been reported (Table I). Consequently no special attempt was made to collect extensive data on this species. Limited data on streptomycin excretion have been collected on one adult and 3 infants to compare recovery rates in the urine with those found in mice, using the same bio-assay procedure.[10]

An adult patient, weighing 160 lb, was given 1,850,000 units of streptomycin subcutaneously in divided doses over a 6-day period. The average daily dose was 4,200 units per kg. The overall recovery of antibiotic in the urine was 75% of the streptomycin administered, but the ratio of units administered to units recovered in the urine varied somewhat during various stages of the study.

In 3 small children receiving 20,000 units of streptomycin per lb (ca 9100 units per kg) per day, intramuscularly, in interrupted doses or by continuous drip, the streptomycin administered which could be accounted for in the urine at any given period during the course of treatment again varied considerably with the individual. The overall recoveries of antibiotic in the urine were 31.5, 45.0 and 45.5% respectively. Although these are lower recoveries than some reported in adults, they fall within the general range found in man.

Conclusions. Whereas streptomycin is absorbed and excreted considerably faster in mice than in man, the percentage recovery of streptomycin in the urine of these 2 species is very similar. Hence, there is no reason to believe that this antibiotic is destroyed in mouse to any greater extent than in man.

Despite the fact that streptomycin is adsorbed on alkaline alumina, streptomycin administered in mixture with alkaline potassium aluminum sulfate is excreted as rapidly in mice as is streptomycin given alone.

15688 P

Proteins with Growth-Promoting Action on Tissue Cells *in vitro*.

Charity Waymouth. (Introduced by A. Fischer.)
From Carlsberg Fund Biological Institute, Copenhagen, Denmark.

Among the many substances which contribute to the effectiveness of embryonic tissue extracts as growth-promoting media for cells *in vitro*, some have been found to be associated with the proteins of the extracts. The ease of inactivation of the extracts by heat and by proteolytic enzymes were early indications that proteins played some role in the effects which result in increasing the area of explanted tissue relative to the area of sister control-cultures.

The lability of the high molecular substances in the embryonic extracts makes it necessary to use only very mild methods in attempts to extract or purify active fractions. It has been found by Fischer and Astrup[1] that a rather high activity was associated with a fraction separated by the method used by Hammarsten[2] for preparation of nucleoproteins. Experiments have now confirmed that such fractions contain the relevant activity. It is, however, not yet certain that the activity necessarily resides in a protein, as distinct from a protein-complex containing nonprotein material, for one of the results of using mild preparative procedures is that labile, loose linkages may not be broken.

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[1] Fischer, A., and Astrup, T., *Pflüg. Arch. ges. Physiol.*, 1943, **247**, 34.

[2] Hammarsten, E., *Hoppe-Seyl Z.*, 1920, **109**, 141.

Examination of some crude nucleoprotein fractions has revealed no correlation between the activity and the proportions of the 2 types of nucleic acid, *i.e.* those containing ribose and desoxyribose. Activity has been found in fractions containing only a trace of desoxyribonucleic acid, so it is concluded that, if a nucleic acid is essential for activity, it must be one of the ribose and not of the desoxyribose type. Ribonucleic acid alone, in the depolymerised form in which it is isolated, does not appear to possess the activity. No method has so far been found for dissociating the ribonucleic acid from the protein, with which it is in firm combination, without denaturing the protein, so it cannot be stated whether the protein moiety alone shows any effect.

A convenient material for studying the active protein fraction of embryonic extracts has been prepared by precipitation of extracts in the cold (—15°C) by a modification of the method used by Hardy and Gardiner[3] and others for delipidation of serum and plasma proteins. Enbryonic extracts are made by mixing equal parts of tissue pulp and Ringer solution, and they therefore contain only material relatively easily extractable from the tissue. Such extracts, and hence also the powders prepared from them by cold-precipitation, are almost completely free from desoxyribonucleic acid. The stable, dry preparations of tissue protein obtained in this way give solutions in saline which possess the activity under discussion.

Examination of solutions of some of the powders so prepared in the Tiselius electrophoresis apparatus revealed that chick embryo preparations contain components of relatively few different mobilities. Preparations from calf embryos were even simpler in this respect, showing, in buffers of 2 different pH values, only a narrow range of mobilities. It was therefore thought to be of interest to examine the sedimentation of such a preparation in the ultracentrifuge; and through the courtesy of Dr. K. O. Pedersen of Upsala, this was done. For comparison with the calf embryo preparation, a powder prepared in exactly the same manner from the muscle of adult cow was also examined. The cow muscle preparation contained, as would be expected, several components, notably those with sedimentation constants (s_{20}) in the region of 1, 5 and 8. Electrophoretically this preparatoin was relatively homogeneous. The embryonic calf preparation, on the other hand, contained only one principal component, with $s_{20} = 4.8$, and corresponding therefore in size with the albumin group of proteins.

Of the low molecular substances precipitated with the proteins by the Hardy and Gardiner technic, a special examination of the phosphorus fractions has been made, since, as in plasma (Waymouth and Davidson[4]), a large proportion of inorganic and acid-soluble phosphorus appears in the final product. The greater part of these fractions could, however, be removed from the extract before precipitation, by dialysis overnight against running tapwater at about 10°C, without affecting the activity of the protein powder upon the growth of the tissue cultures.

These preliminary experiments have demonstrated that a type of growth-stimulating activity is associated with a protein fraction containing ribonucleoprotein, and possessing, for a material prepared by such a crude procedure, a surprisingly high degree of homogeneity in electrophoretic migration and in ultracentrifugal sedimentation. Growth-promoting activity was also found in adult tissue preparations made in a similar way, and it is thought that the type of substance responsible for the effect may be widely distributed in tissue cells, though masked in adult tissue extracts by the relatively higher proportion of nonactive components.

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[3] Hardy, W. B., and Gardiner, S., *J. Physiol.*, 1910, **40**, lxviii.

[4] Waymouth, C., and Davidson, J. N., *Biochem. J.*, 1946, **40**, proc.

15689

Changes in Concentration of Enzymes in Pancreatic Juice After Giving Insulin.*

J. E. Thomas and J. O. Crider.

From the Department of Physiology, Jefferson Medical College.

Babkin[1] has recently reviewed the literature dealing with the effects of hypoglycemia and hyperglycemia on the external secretory function of the pancreas, hence only a brief summary of the conclusions to be drawn from previous experimental work will be given here. In cross-circulated dogs hypoglycemia of the head diminishes and hyperglycemia augments the output of fluid and enzymes from the pancreas (La Barre and Destrée[2-4]). In rabbits hypoglycemia decreases the output of enzymes from the pancreas through a central action and hyperglycemia increases the output through a peripheral action (Baxter,[5] Hebb[6]). Also in rabbits the normal "trophic" effect (increase in enzyme output) of vagus stimulation is reversed by hypoglycemia (Hebb[6]). Experiments on human subjects have given quite different results from those obtained on anesthetized animals. Insulin hypoglycemia stimulates the flow of pancreatic juice in man (Okada[7]) but not if gastric juice is excluded from the intestines (Frisk and Welin[8]). The same is true of fistula dogs (Scott *et al.*[9]) When given with secretin, insulin increases the output of pancreatic enzymes in man (Lagerlof and Welin[10]).

Apparently no information is available regarding the effect of insulin-induced hypoglycemia on the output of pancreatic enzymes in unanesthetized experimental animals. This report is intended as a contribution to the study of that problem.

Methods. The experiments were performed on 3 dogs having tubulated gastric and duodenal fistulas with the duodenal fistula opposite the main pancreatic duct. Pancreatic juice was collected through a temporary glass cannula inserted into the duct via the duodenal fistula.[9,11] The stomach was drained with the aid of suction through a double drainage tube passed through the gastric fistula all the way to the pylorus. The drainage tube was made up of a rather large soft rubber outer tube within which was a much smaller inner tube. Both tubes had numerous perforations near the pyloric end. The inner tube was connected through a trap to a water-operated vacuum pump.

Because insulin alone does not stimulate pancreatic secretion in the dog,[9] secretin was given by continuous intravenous injection throughout each experiment by means of a motor-driven pump. The rate of secretin injection was adjusted to give an estimated 3 to 4 cc of pancreatic juice in 10 minutes but these limits were frequently exceeded in practice.

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* We are indebted to Dr. M. H. F. Friedman for making the determinations of tryptic activity and to Dr. Friedman and to Wyeth, Inc., for adequate supplies of secretin. We are also indebted to Prof. Abraham Cantarow in whose laboratory the blood sugar determinations were made.

[1] Babkin, B. P., *Secretory Mechanism of the Digestive Glands*, Hoeber, New York, 1942.

[2] †La Barre, J., and Destrée, P., *C. R. Soc. Biol. Paris*, 1928, **98**, 1237; *Ibid.*, 1928, **98**, 1240; *Ibid.*, 1928, **99**, 1056; *Ibid.*, 1928, **99**, 1874; *Ibid.*, 1929, **101**, 147; *Ibid.*, 1935, **110**, 1177.

[3] † La Barre, J., *C. R. Soc. Biol. Paris*, 1928, **99**, 1053; *Am. J. Physiol.*, 1930, **94**, 117.

[4] † Destrée, P., *C. R. Soc. Biol. Paris*, 1930, **104**, 1038.

† Cited by Babkin.[1]

[5] † Baxter, S. G., *Quart. J. Exp. Physiol.*, 1932, **21**, 355.

[6] †Hebb, C. O., *Quart. J. Exp. Physiol.*, 1937, **26**, 339.

[7] †Okada, S., *Nagoya J. med Sci.*, 1933, **7**, 91.

[8] † Frisk, A. R., and Welin, G., *Acta med. scand.*, 1937, **91**, 170.

[9] Scott, V. Brown, Collingnon, U. J., Bugel, H. J., and Johnson, G. C., *Am. J. Physiol.*, 1941, **134**, 208.

[10] †Lagerlof, H., and Welin, G., *Acta med. scand.*, 1937, **91**, 397.

[11] Hart, Wm. M., and Thomas, J. E., *Gastroenterology*, 1945, **4**, 409.

The dogs were fed once daily and were used for experiments before feeding, when the stomach was generally empty. If any food residues remained they were removed through the fistula before starting the experiment.

Pancreatic juice was collected continuously and divided into separate samples, each collected during a 10-minute interval. After a control period during which at least 5 10-minute samples were collected with secretin alone, insulin was given intravenously in doses ranging from 7.5 to 20 units (total dose). The experiment was then continued for one hour or longer without further modification. Blood samples were collected from a leg vein at intervals throughout each experiment. Blood sugars were determined by the Benedict method.[12]

The volume and specific gravity of each 10-minute sample of pancreatic juice were measured. Specific gravity was determined by weighing the juice in a 1 ml specific gravity bottle at room temperature. The control samples and the samples collected after giving insulin were then poured into separate containers and the proteolytic activity determined on each group of pooled samples. The first sample collected and any additional samples obtained while adjusting the rate of secretin injection were excluded from the control pool.

Results. Sixteen reasonably satisfactory experiments were performed. Four experiments were discarded because of technical difficulties, the most common being the appearance of excessive amounts of acid in the duodenal contents. Only those experiments were discarded in which, in our judgment, the technical failures materially influenced the results.

Blood Sugars. The range of control blood sugar concentrations was approximately the same in the 3 dogs used. The highest control value was 95 mg per 100 ml and the lowest 51 mg. Only 3 were above 70 mg and 3 were below 60 mg. The doses of insulin were sufficient to lower the blood sugar to between 30 and 40 mg in most instances. In 2 experiments, both in the same animal (2-46) the blood sugar fell to below 30 mg. Restlessness, dilation of the pupils, increase in pulse rate and, occasionally, drowsiness were observed in association with hypoglycemia. Blood sugar values are recorded along with other data in Table I.

Changes in Specific Gravity of Pancreatic Juice. Specific gravity was used as an index of the nitrogen content of the pancreatic juice with which it has a linear relation.[13] The nitrogen, in turn, is believed to vary with the enzyme content.[14] After giving insulin the specific gravity invariably increased. The increase was generally evident in the first 10-minute sample collected after giving insulin and reached a maximum within 30 to 40 minutes. The subsequent decline was gradual and seldom reached the control level during the one to 2 hours that the observations were continued. Detailed data are given in Table I and a typical experiment is illustrated in Fig. 1. The significance of the changes in specific gravity will be more evident if the estimated values for average total nitrogen before and after giving insulin are compared. Increases of well over 100% of the control value for total nitrogen are common among the average values after insulin. The average values, however, fail to reveal the full effect of insulin since they include data on samples collected before the response was fully developed. In this situation the maximum values are more significant.

Changes in Tryptic Activity. Tryptic activity is expressed in Table I in terms of the velocity constant, k, as determined by the rate of decrease in turbidity of a colloidal suspension of coagulated egg white.[15,16] It is evident that in the 10 experiments in which enzyme determinations were made the tryptic activity increased with increasing specific gravity in all but 2. In one of these

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[12] Benedict, S. G., *J. Biol. Chem.*, 1920, **83**, 165; 1928, **76**, 457.

[13] Crider, J. O., and Thomas, J. E., *Am. J. Physiol.*, 1944, **141**, 730.

[14] Babkin, B. P., and Tichomirov, N. P., *Z. f. physiol. Chem.*, 1909, **62**, 468.

[15] Friedman, M. H. F., in press.

[16] Riggs, B. C., and Stadie, W. C., *J. Biol. Chem.*, 1943, **150**, 463.

TABLE I.

Effect of Insulin on Pancreatic Secretion Induced by Secretin.

		Control period					After giving insulin (first hour)						
Dog No.	Date 1946	Avg vol. of 10 min, samples, cc	Specific gravity, avg	Avg total N (estimated) mg/cc	Trypsin, ‘‘k’’	Blood sugar, mg/100 cc	Dose of insulin, units	Avg vol. of 6 10-min. samples, cc	Specific gravity Max.	Specific gravity Avg	Avg total N (estimated) mg/cc	Trypsin, ‘‘k’’	Min. blood sugar, mg/100 cc
1-44	5/22	4.5	1.0108	1.96	0.143	77.5	20	3.2	1.0156	1.0133	3.44	0.170*	35
,,	31	4.4	1.0107	1.90		63.7	15	4.3†	1.0167	1.0144	4.10		31
,,	6/ 4	3.9	1.0119	2.61		69	15	5.3	1.0177	1.0158	4.93		33
,,	17	3.9	1.0114	2.32	0.155	68.5	15‡	3.7	1.0154	1.0138	3.75	0.101§	50
,,	7/30	2.8	1.0118	2.55		95.5	15	2.3	1.0184	1.0160	5.05		49
1-46	6/ 3	4.8	1.0118	2.55		51.5	20	5.1‖	1.0156	1.0141	3.93		31
,,	7	3.5	1.0109	2.02	0.250	68	10	4.5	1.0145	1.0134	3.51	0.425	36.5
,,	19	5.1	1.0109	2.02	0.110	66	10	3.7	1.0132	1.0121	2.73	0.105	40
,,	24	3.4	1.0092	1.01	0,05	69	7.5	6.3	1.0113	1.0102	1.66	0.09	51
,,	7/ 1	3.4¶	1.0098	1.36		58	15	4.0	1.0135	1.0119	2.61		34
2-46	6/ 5	2.8	1.0104	1.72	0.141	86	20	3.8	1.0192	1.0156	4.81	0.450	22.5
,,	10	4.9	1.0112	2.20	0.260	66	10	3.6	1.0152	1.0126	3.03	0.310	34.5
,,	18	4.2	1.0100	1.48	0.120	52	10	2.6	1.0159	1.0125	2.97	0.19**	28
,,	21	3.0	1.0096	1.25	0.075	60.5	15††	4.5	1.0116	1.0105	1.78	0.120	55
,,	,,						5‡‡	5.4	1.0147	1.0131	3.33	0.260	30
,,	7/ 8	2.3	1.0111	2.14		60.5	15	2.0	1.0178	1.0159	4.99		36
,,	25	4.4	1.0099§§	1.43	0.115§§	61.5	7.5	5.4	1.0115	1.0101	1.54	0.170	31

* 2 samples. Avg sp. g. 1.0114.

† Traces of acid in duodenum.

‡ Injected subcutaneously.

§ See text.

‖ 2 samples omitted because of change in rate of secretin injection.

¶ Acid in duodenum at start, none later.

** 5 samples. Avg sp. g. 1.0138.

†† Injected subcutaneously.

‡‡ Second dose of insulin; given intravenously.

§§ Includes 4 samples excluded in estimating volume because of difference in rate of secretin injection.

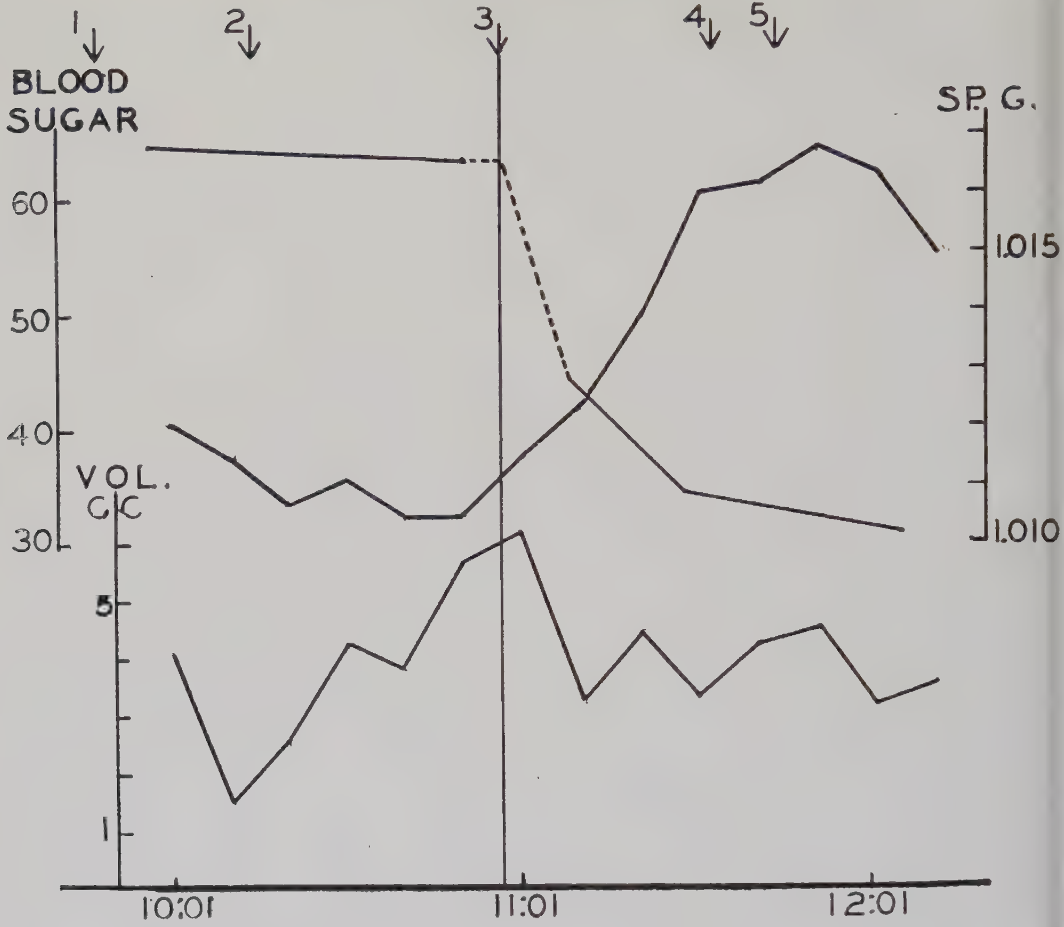

FIG. 1.

Curves showing, in order from above downward, blood sugar, specific gravity of pancreatic juice, and volume of juice in a typical experiment with insulin on Dog 1-44, May 31, 1946. Meaning of arrows:

1. Secretin injection started.
2. Rate of secretin injection increased from 0.6 mg to 1.0 mg in 10 minutes.
3. Fifteen units of insulin (Mulford) given by vein.
4-5. Traces of acid in duodenal contents.

(note "d" in Table I), the experiment was on a dog (1-44) in which discrepancies between tryptic activity, and total nitrogen as determined by the Kjeldahl method, were common. Such discrepancies were rare or did not occur in a considerable number of other dogs that we have studied and we believe this dog to be unique in this respect. In the other instance (Dog 1-46, 6/19/46) the difference is small and probably within the range of experimental error.

Effects on the Volume of Pancreatic Juice. In 8 of the experiments summarized in Table I the average volume of the 10-minute samples of pancreatic juice secreted during the first hour after insulin was greater than during the control period. In 5 it was less and in 3 there was practically no change (less than 0.5 cc). The differences in either direction seldom exceed the normal range of variation and are clearly not significant. However, in a few experiments there was a definite decrease in volume after insulin which, when it occurred, became more evident toward the end of the experiment.

Discussion. According to the available evidence the changes in visceral function following administration of insulin are caused by stimulation of the vagus centers through the influence of hypoglycemia. Our experiments provide no additional evidence for or against that view. The occurrence of restlessness and tachycardia in dogs, convulsions in other animals, and of sweating and convulsions in the human indicates that the stimulation is general and is not confined to the vagus or the parasympathetic centers. The increased output of epinephrin which tends to counteract the fall in blood sugar may be taken as a specific indication of stimulation of thoracolumbar sympathetic mechanisms.

In such a situation the effect on the pancreas, which receives both excitatory and inhibitory innervation, could hardly be predicted. It would doubtless represent a summation of antagonistic influences and would probably be readily modified by experimental conditions such as anesthesia and operative trauma. It is not surprising, therefore, that our results in unanesthetized dogs are quite opposite to those previously reported on anesthetized, operated animals. Since they agree with the results obtained in human experiments they probably represent the normal reaction of the pancreatic secretory mechanism to the complex situation resulting from acute hypoglycemia.

The inhibitory effects of hypoglycemia were manifest in some of our experiments only as a late decrease in volume of pancreatic juice secreted in response to a constant stimulus (secretin). Babkin[1] has suggested that these effects may be due to inability of the pancreas to function normally without an abundant supply of carbohydrate for its metabolic needs. In our experiments the volume, when low, could be promptly restored to normal by slightly increasing the rate of secretin injection. The specific gravity of the juice remained high in these circumstances. Clearly the pancreas was still capable of normal function when adequately stimulated. The decreased response to secretin in these experiments could be attributed to vascular changes or to preponderance of the inhibitory innervation.

Summary and Conclusions. Specific gravity and tryptic activity of dog's pancreatic juice secreted in response to continuous injection of secretin are increased following administration of insulin. The average volume secreted during the first hour after giving insulin is not significantly affected. Inhibitory effects of hypoglycemia described as occurring in anesthetized, operated animals are not readily demonstrated in unanesthetized dogs.

15690

Some Effects of Desoxycorticosterone Acetate on Mice Irradiated with X-rays.*

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Total body irradiation with X-rays produces fatty changes in the livers of irradiated animals.[1] We have recently demonstrated that the administration of desoxycorticosterone acetate protects the livers of mice irradiated with various lethal doses of X-rays against these fatty liver changes.[2] It was noted in addition that there was a decrease in the mortality rates produced by the previously used X-ray doses. It is the purpose of this paper to analyze these phenomena

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* Aided by grants from the John and Mary R. Markle Foundation, New York, and the Schering Corporation, Bloomfield, N.J.

[1] Ellinger, F., *Radiology*, 1945, **44**, 241.

[2] Ellinger, F., *Science*, 1946, **104**, 502.

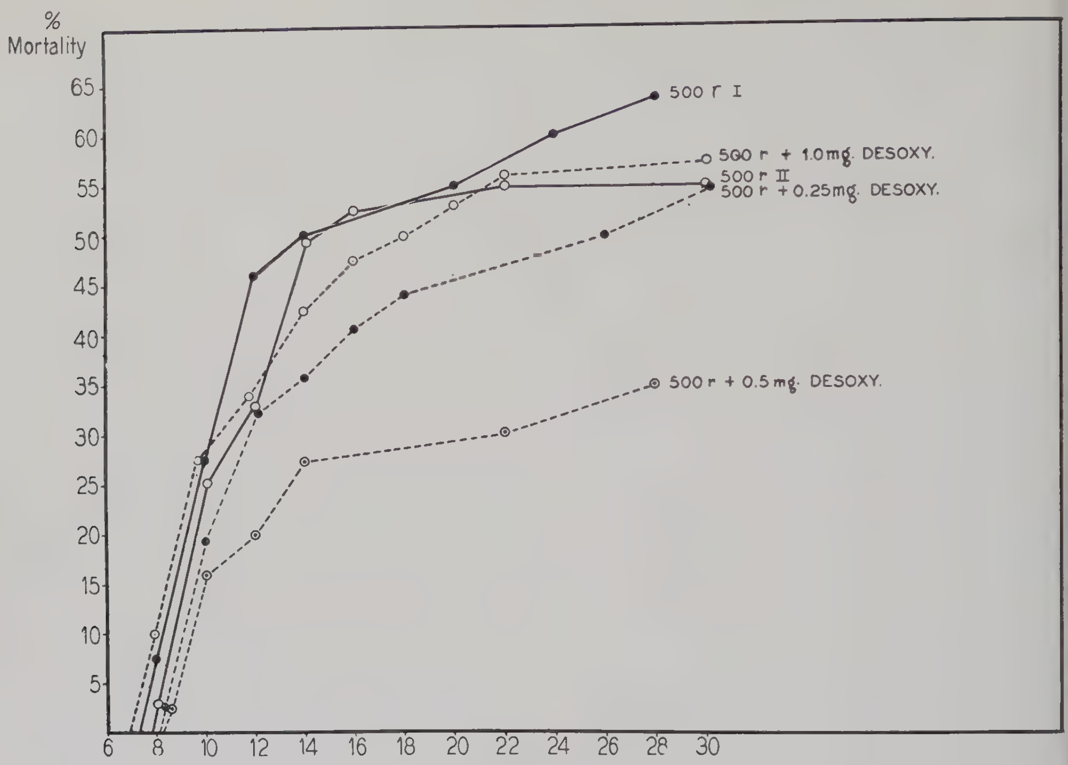

FIG. 1.

Reduction of the mortality rate produced by 500 r/air of X-rays in mice by administration of daily doses of desoxycorticosterone acetate of 0.25, 0.5, and 1.0 mg respectively. Abscissa: days after exposure to X-rays. Ordinate: % mortality.

in more detail.

Methods. A total of 166 white male Swiss mice 22 g ± 15% in weight was used, 62 of these received total body irradiation with X-rays only, while 104 animals received similar irradiation and desoxycorticosterone. All mice received 500 r/air in one exposure (HVL 0.75 mm Cu). This dose represents the LD_{50}. The technic of irradiation was the same as previously described.[3]

Daily doses of desoxycorticosterone acetate[†] in sesame oil of either 0.25, 0.5 or 1.0 mg were administered intramuscularly 6 times weekly over a period of 10-18 days. The total doses varied between 2.5 and 13.0 mg.

The effect on mortality rate, fat content of livers and radiation changes in other organs was studied at death of the animals. Survivors were observed 28-40 days, in some instances up to 240 days.

The fat content of livers was studied using sections stained with Sudan III, with the following grading:

0	No sudanophile fat.
+	Traces of fat, as occasionally seen in non-irradiated livers.
++	Increased amount of fat with definite arrangement around central vessel.
+++	Considerable increase in fat.
++++	Fat making up an entire lobulus.

The arithmetical mean of these various grades of sudanophile fat was used for the quantitative evaluation of the histological changes, and called the "fat index."

Results. A graphic presentation of our experiences with various doses of desoxycorticosterone on the lethal effect produced by 500 r/air of X-rays, given as total body irradiation in one exposure, is shown in Fig. 1. It might be noted that desoxycorticosterone

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[3] Ellinger, F., *Radiology*, 1945, **44**, 125.

[†] We are greatly indebted to Dr. E. Henderson of the Schering Co. for generously supplying us with desoxycorticosterone acetate.

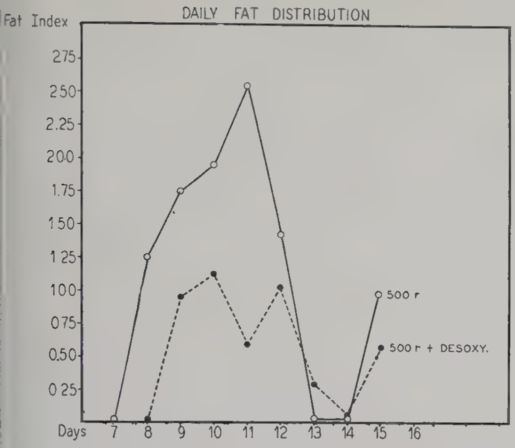

Fig. 2.

Influence of treatment with desoxycorticosterone on the appearance of sudanophile fat in the livers of irradiated mice. Abscissa: Days after exposure to X-rays. Ordinate: Arithmetical mean of the arbitrary grades of sudanophile fat (fat indices) for each day.

decreases the lethal effect of this X-ray dose.

From our previous studies it was inferred that there might be a quantitative relationship between the dose of desoxycorticosterone and the decrease in mortality rate produced by the X-rays.

Our data on the influence of desoxycorticosterone on the mortality rate have therefore been broken down into 3 groups for a closer analysis of this phenomenon: (a) mice receiving 0.25 mg daily (36 animals), (b) mice receiving 0.5 mg daily (34 animals), (c) mice receiving 1.0 mg daily (34 animals). Furthermore, the total data concerning the mortality rate produced by irradiation of mice with 500 r/air not treated with desoxycorticosterone has been divided into 2 groups: 500 r I and II consisting of 35 and 27 animals respectively, in order to demonstrate the variation in the mortality rate of solely irradiated mice. The graph demonstrates a slight variation in the mortality curves of the 2 groups of mice which received irradiation only. Administration of daily doses of 0.25 mg of desoxycorticosterone decreases the lethal effect of the X-ray dose beyond the variation observed in untreated irradiated mice. The decrease in mortality rate is still more marked in the group of mice which received 0.5 mg of desoxycorticosterone daily. The administration of 1.0 mg, how-

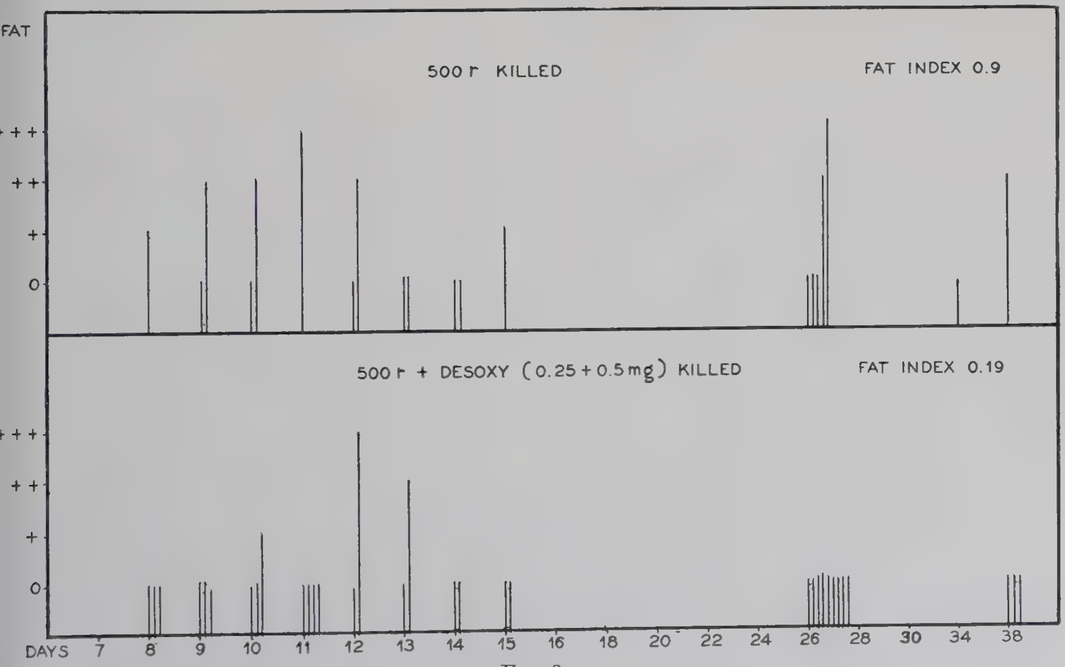

Fig. 3.

A comparison of the fat content of livers in solely irradiated and irradiated and desoxycorticosterone treated animals killed at various days, also indicates reduction in the content of sudanophile fat in the desoxycorticosterone-treated group. Each column represents the findings in one mouse. Abscissa: Days after exposure. Ordinate: Arbitrary grades of sudanophile fat.

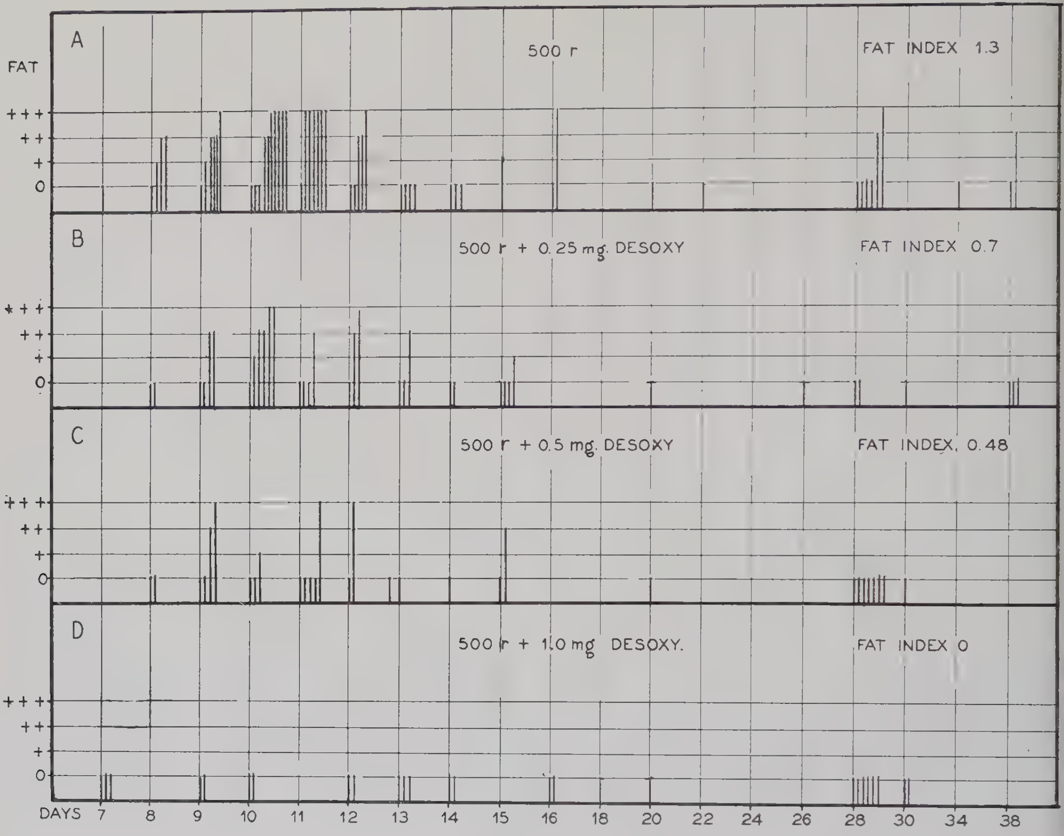

Fig. 4.

Analysis of the influence of the size of the daily dose of desoxycorticosterone acetate on the appearance of sudanophile fat of the livers of irradiated mice, indicates a graded effect of desoxycorticosterone.

ever, does not change the mortality rate. The mice receiving the largest daily doses evidently were suffering from an intoxication produced by these large amounts of desoxycorticosterone, which manifested itself in a ragged fur, sometimes loss of fur and considerable emaciation of the animals.

Our observations concerning the liver changes found in both the untreated irradiated and the irradiated and desoxycorticosterone-treated mice are presented in Fig. 2. This graph shows that the decrease in mortality rate produced by the administration of desoxycorticosterone is accompanied by a marked and statistically valid decrease in the formation of sudanophile fat in the **livers** of irradiated and desoxycorticosterone-treated animals.

Fig. 2 confirms previous observations[1] that the occurrence of sudanophile fat in irradiated livers proceeds in waves with peaks at various times after cessation of irradiation.

Recognition of this fact raises the question, whether the observed reduction in the occurrence of sudanophile fat in the irradiated and desoxycorticosterone-treated animals might not be due to the fact that death in these animals occurs at later dates, where the manifestation of sudanophile fat is less pronounced.

To answer this question animals have been killed at various dates after irradiation in both groups. Fig. 3 shows our observations. The data, though not statistically relevant, appear significant, showing a marked reduction in the amount of sudanophile fat in the desoxycorticosterone-treated group, when compared with the finding in the untreated irradiated mice. The fat indices are 0.19 and 0.9 respectively.

In Fig. 4, finally, data on the effect of desoxycorticosterone on the appearance of sudanophile fat are arranged according to the size of the daily doses of desoxycorticosterone. Section A of Fig. 4 shows the results in the untreated irradiated mice. Section B in those which received 0.25 mg of desoxycorticosterone daily, Section C of those which received 0.5 mg and Section D of those which received 1.0 mg daily. The fat indices are 1.3, 0.7, 0.48 and 0.00 respectively.

The trend of Fig. 4 corresponds to the observations concerning the influence of various daily doses of desoxycorticosterone on the mortality rate. It is very interesting to note that the administration of daily doses of 1.0 mg are able to suppress the appearance of sudanophile fat in the livers completely, although these doses do not reduce the mortality rate.

No significant changes in the effect of the X-ray on spleen and bone marrow was observed with any of the used doses of desoxycorticosterone.

Discussion. The data presented in this paper confirm the previously described protective action of desoxycorticosterone acetate against X-ray-induced liver changes. They establish a parallelism between the reduction in sudanophile liver fat and the decrease in mortality rate in the irradiated and desoxycorticosterone-treated animals.

The analysis of the phenomena presented reveals that both effects within certain limits are dependent on the size of the daily dose of desoxycorticosterone.

The observation of a graded action of desoxycorticosterone with respect to its protective power against X-ray-induced liver damage, seems to indicate a definite antagonistic effect to the processes leading to the appearance of sudanophile fat in the liver.

We have recently adduced the evidence[1] which makes it highly probable that the occurrence of fatty changes in the livers of irradiated animals is the result of an indirect action of the rays by release of tissue breakdown products, histamine-like in character, most probably histamine itself.

Since desoxycorticosterone is known to counteract certain histamine effects[4] it appears highly probable that its protection against radiation-induced liver changes is based on this known histamine antagonism.

In favor of this explanation of the effect of desoxycorticosterone is the observation that desoxycorticosterone leaves the radiation effects on spleen and bone marrow unchanged. These effects are considered as predominantly the result of the direct destructive action of the X-ray in these tissues.[3]

The evidence presented in this paper indicates the possibilities of closer analysis of radiation effects by pharmacological means.

Finally, these data provide a pharmacological basis which justifies the use of desoxycorticosterone in the treatment of radiation sickness, a general intoxication of the irradiated patient, caused by the release of tissue break-down products. While our data indicate in a general way the usefulness of desoxycorticosterone for the treatment of radiation sickness, these observations should not be interpreted as indicating optimal doses for therapeutic purposes.

Summary. 1. A reduction in mortality rate of mice irradiated with X-ray by the use of desoxycorticosterone acetate has been demonstrated. 2. The reduction in mortality rate has been correlated with the protective action of desoxycorticosterone against radiation-induced liver changes. 3. It has been shown that reduction in mortality rate and protection against radiation-induced liver changes are dependent, within limits, on the size of the daily dose of desoxycorticosterone. 4. It has been suggested that the graded effect of desoxycorticosterone is possibly due to an antagonism to the indirect action of the X-rays which consists in the release of histamine-like substances from the irradiated tissues. 5. The data presented are proposed as a pharmacological basis which justifies the clinical use of desoxycorticosterone in the treatment of radiation sickness.

The author wishes to express his gratitude to Dr. A. L. L. Bell, Director of the Department of Radiology, Long Island College of Medicine, for his kind interest in and support of these investigations.

Valuable technical assistance has been rendered by Miss Jean Bernstein.

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[4] Swingle, W. W. and Remington, J. W., *Physiol. Rev.*, 1944, **24**, 89.

15691

Quantitative Measurement of Growth of *Mycobacterium tuberculosis.* Effect of Streptomycin.*†

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Quantitative estimation of the growth of *Mycobacterium tuberculosis* has been seriously handicapped by lack of simple, accurate, and safe methods of measurement. For this reason, the determination of the normal culture cycle of this organism, the rate of growth, and the *in vitro* effects of growth-promoting and growth-inhibiting substances has been difficult. Visual approximation of growth and weighing of pellicles with all the concomitant danger due to handling of the culture have been the only available, though unsatisfactory, methods. These were dangerous, or they involved technical difficulties and inaccuracies, as pointed out by Mueller.[1]

Recently, Youmans[2] measured the normal culture cycle, growth rate, and generation time of the H37Rv strain of the virulent human type tubercle bacilli by means of micro-Kjeldahl nitrogen determinations. He suggested the use of this procedure in the evaluation of the efficacy of chemotherapeutic agents upon the organism of tuberculosis. Because of the complexity of this test, its use for mass determinations may be questioned.

Dubois[3] suggested the possibility of making use of nephelometric measurements in establishing the value of the oil of chaulmoogra and its derivatives on the growth of avian bacilli in Kirchner's medium.[4] Homogeneous growth in this medium, contrary to the usual result obtained with cultures of mycobacteria, was reported.

Dubos,[5] using a modification of the Kirchner formula, developed a medium in which both the pathogenic and the saprophytic varieties of mycobacteria produce a diffuse type of growth. At times, human bacilli form microscopic clumps of loosely packed cells, which can be readily dispersed by moderate shaking to form a uniformly turbid suspension. Such uniformly diffused growth facilitates accurate turbidimetric measurements.

Since none of the existing procedures was satisfactory for safe and accurate determination of growth of mycobacteria, advantage was taken of the uniform turbidity produced in Dubos' medium to develop a suitable method for estimating the growth of these organisms. This method found special application in the study of the effect of streptomycin upon the growth of this organism.

Procedure. Stock cultures of *M. tuberculosis avium* (ATCC No. 7992), an avirulent strain of *M. tuberculosis var. hominis* No. 607, a virulent strain of *M. tuberculosis var. hominis* H37Rv, and a streptomycin-resistant strain of *M. tuberculosis var. hominis* H37Rv obtained from Dr. G. P. Youmans were maintained on glycerol-nutrient agar slants and in Dubos' medium.[5] For the growth of virulent human varieties, 0.2% bovine serum albumin (fraction V) was added to the medium. The ages of the cultures varied depending on their rate of growth, namely, 7 days for *M. avium* and *M. tuberculosis* No. 607 and 3 to 5 weeks for *M. tuberculosis* H37Rv and H37Rv streptomycin-resistant.

For purposes of inoculation, a homogeneous suspension of cells was made in a simplified modification of Dubos' medium, which was incapable of supporting significant growth and consisted only of the salt mixture plus

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* Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology.

† Partly supported by a grant made by the Albert and Mary Lasker Foundation of New York.

[1] Mueller, J. H., *J. Bact.*, 1935, **29**, 383.

[2] Youmans, G. P., *J. Bact.*, 1946, **51**, 703.

[3] Dubois, A., *Ann. Soc. belge de Med. Trop.*, 1944, **24**, 1.

[4] Kirchner, O., *Zentral. f. Bakteriologie,* 1932, I Orig., **124**, 403.

[5] Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

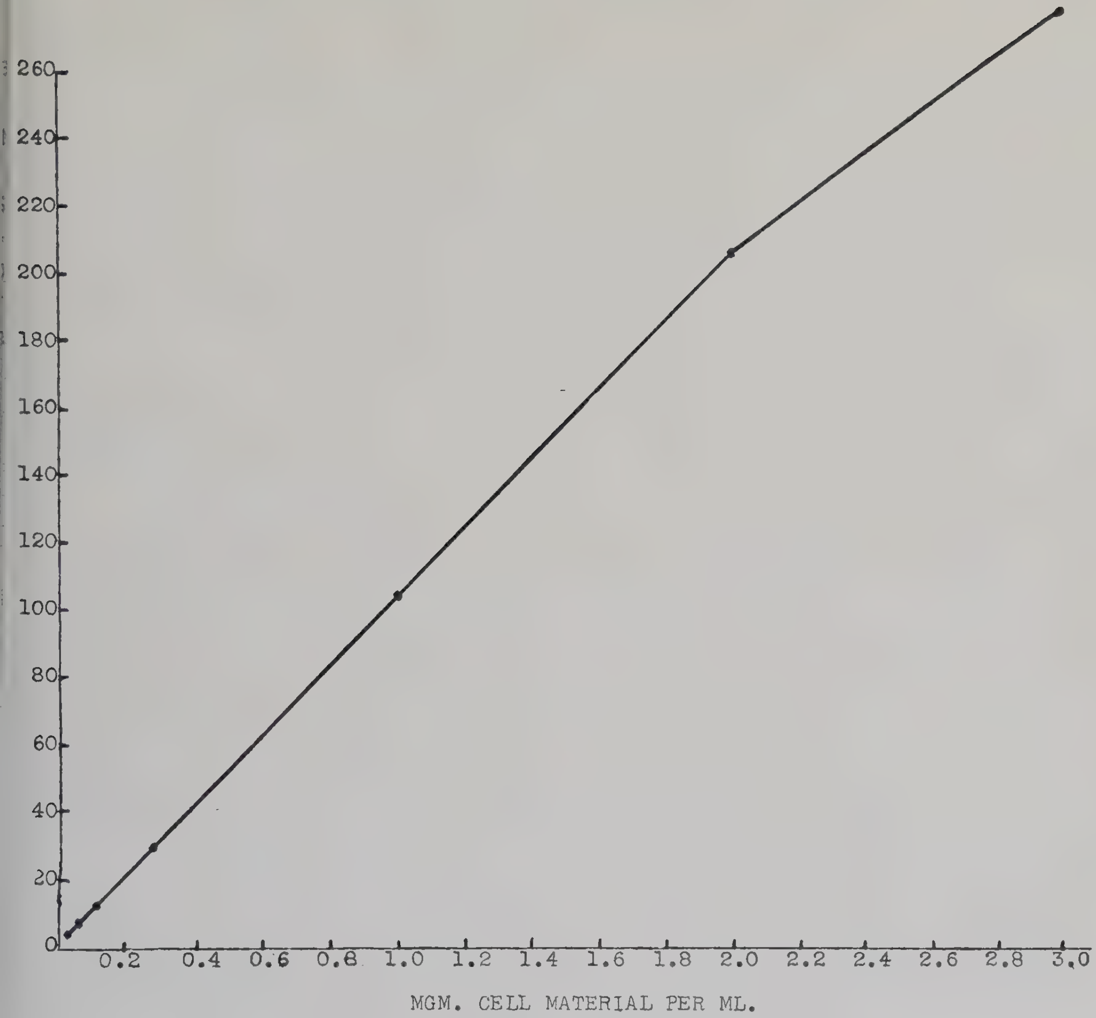

FIG. 1.

Turbidimetric Measurement of Suspensions of *Mycobacterium tuberculosis* No. 607.

Tween 80.‡ The purpose of this Tween-salt solution was to avoid, as much as possible, the introduction of nutrients when inoculating nutritionally-deficient media.

The suspension was prepared as follows: Growth from an agar slant was added to a small amount of the Tween-salt mixture and shaken with sterile glass beads. This resulted in a homogeneous suspension which could be diluted to the desired density. If cultures grown in Dubos' medium were used, they were diluted or were used undiluted, depending upon their density. Turbidimetric readings of suspensions containing weighed amounts of cells have shown that 1 mg of cells (wet weight) per milliliter of fluid formed a suspension which gave turbidimetric readings of 100-105 on a Klett-Summerson colorimeter. A certain amount of growth of the organism was suspended in the medium and adjusted to give a reading of 100. Further dilution was made with the Tween-salt solution until the required concentration of cells was obtained.

The experiment was now set up by seeding the media with 4.5 ml portions of a suspension of bacterial cells in test tubes. For controls, 5-ml amounts were used; for the study of the effect of streptomycin, 0.5-ml portions of the various concentrations of drug

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‡ Tween 80 is a polyethylene derivative of sorbitan monooleate and acts as a detergent in this medium.

TABLE I.
Influence of Inoculum on Rate of Growth of *Mycobacterium tuberculosis* No. 607.*

	Concentration of cells, mg/ml				
	10^{-3}	10^{-5}	10^{-7}	10^{-9}	10^{-10}
Time of incubation, hr	Turbidimetric readings (in logs)				
24	9	7	0	0	0
48	26	7	0	0	0
72	44	34	8	0	0
96	60	54	31	8	0
184	106	100	83	72	0
330	150	159	138	153	0

* Dubos' medium without enrichment with dextrose, vegex, and albumin.

were added to 4.5 ml of the seeded medium. The cultures were incubated at 37°C, and turbidimetric readings were made at frequent intervals. In media with extremely low nutritive value, some firm clumps of cells were formed after 10 to 21 days of incubation, depending on the rate of growth of the culture. No clumping was observed in Dubos' medium even after 6 weeks' incubation.

The tubercle bacilli can be grown on fairly simple media. Dubos' medium, consisting of KH_2PO_4, $Na_2HPO_4 \cdot 12H_2O$, $MgSO_4 \cdot 7H_2O$, sodium citrate, Tween 80, an enzymatic digest of casein, dextrose, vegex (a vegetable extract), and bovine serum-albumin, is rather complex and supports abundant growth of the human pathogenic strains of mycobacteria as well as of the saprophytic forms. However, considerable growth can still be obtained if all complex organic materials are omitted and ammonium salt is used as a source of nitrogen with the citrate as the sole source of carbon. Thus the above medium can be simplified so as to be made synthetically, and still remain adequate for growth of the organism. By the use of this or similar modifications, it was possible to study the physiology of the tubercle bacilli and to investigate the effect of streptomycin on the metabolism of the organism.

In preliminary experiments, bacterial suspensions were prepared for purposes of inoculation using weighed amounts of cells. The uniform turbidity of these suspensions made possible the construction of a standard curve for the determination of the weight of the cell material in terms of turbidity readings. This is shown in Fig. 1, where the weight of the cells ranging from 0.01 mg/ml to 2.0 mg/ml was plotted against turbidimetric readings as given on the scale of the Klett-Summerson colorimeter. Readings above 2.0 mg/ml diverged from a straight line, whereas those below 0.01 mg/ml could not be accurately measured. Two advantages of such a procedure are obvious, namely, the ease of correlating turbidity with the weight of cell material, and the elimination of undue handling of the culture.

If a standard curve is made based on the plate counts of viable bacteria in a suspension, the value of the turbidimetric readings

TABLE II.
Influence of Nitrogenous Materials on the Rate of Growth of *Mycobacterium tuberculosis var. hominis* H37Rv.

Concentration of cells in mg/ml, 5 × 10^{-3}				
Constituent of medium	NH_4 citrate, 0.2%	Asparagine, 0.2%	Tryptophane, 0.1%	Dubos' medium (albumin)
Time of incubation, hr	Turbidimetric readings (in logs)			
24	0	0	2	0
72	0	2	8	3
120	0	4	13	16
244	9	16	20	56
334	11	19	26	85
507	11	13	34	244

TABLE III.
Influence of Nitrogenous Materials on the Bacteriostatic Action of Streptomycin. *Mycobacterium tuberculosis* No. 607*.

Source of nitrogen	Streptomycin, µg/ml	Incubation in hr					Final pH
		24	48	72	187	312	
		Turbidimetric readings (in logs)					
NH_4 citrate, 0.2%	0.4	0	17	27	27	67	6.20
	0.2	0	11	27	25	67	6.20
	0.1	1	16	27	30	72	6.20
	0	1	14	29	30	60	6.20
Asparagine, 0.2%	0.4	0	0	0	0	5	7.88
	0.2	0	0	0	30	45	7.92
	0.1	0	16	37	35	31	8.05
	0	2	25	38	37	85	8.06
Casein digest, 0.2%	0.4	0	0	0	0	0	8.20
	0.2	2	2	3	6	6	8.18
	0.1	2	16	34	83	108	8.30
	0	3	13	43	113	154	8.40
Beef extract, 0.3%	0.4	0	0	0	52	155	7.88
	0.2	1	10	25	96	157	8.38
	0.1	4	27	48	150	165	8.48
	0	5	31	55	165	150	8.45

* The media were seeded with 10^{-3} mg cells/ml.

may be extended to correlate turbidity measurements with the number of viable cells. A series of 10 determinations were made with 7-day-old cultures of *M. tuberculosis* No. 607 and of *M. avium* on glycerol-nutrient agar. Consistently reproducible counts of viable cells were obtained, the numbers of viable organisms being 50.6 million cells for *M. tuberculosis* and 59.5 millions for *M. avium* per milligram, with a turbidity reading of 101, with 1 mg of cell material per milliliter. When determining numbers of viable organisms, certain limitations must be kept in mind. First, at least on solid medium, it is often difficult to initiate growth with small numbers of tubercle bacilli; wide variation in results may, therefore, occur. Secondly, optical densities are proportional to the numbers of bacteria only where organisms of the same size or size distributions occur.[6] When grown in the presence of antibiotic agents, the organisms frequently assume elongated shapes, increasing in volume rather than dividing into separate components. In comparing cultures grown in the presence and in the absence of antibiotic agents, the optical density is a measure of the relative volumes or masses rather than of the numbers of bacteria. Even with these limitations, turbidimetric readings were found to permit satisfactory interpretation of results once the relationship between turbidity, weight of cells, and numbers of viable organisms had been established.

Experimental Results. The application of the turbidimetric method for the quantitative estimation of the growth of tubercle bacilli is demonstrated in several experiments. Table I shows the influence of inoculum on the rate of growth of *M. tuberculosis* No. 607 in a modification of Dubos' medium. During the first 96 to 184 hours, the rate of growth was dependent upon the size of the inoculum; then growth appeared to proceed at a more or less uniform rate regardless of the initial concentration of the cells, as indicated by the readings at 330 hours. Similar results were obtained with *M. avium* and with the streptomycin-resistant and -sensitive strains of *M. tuberculosis* H37Rv. In this case, as in all other media used for the growth of tubercle bacilli, the strains pathogenic to man grew at a much slower rate than the saprophytes. The data also agree with the plate counts of viable bacteria, growth taking place with 10^{-9} mg of cell material per milliliter but not with 10^{-10}. According to the actual count, 10^{-7} mg of cell material should contain 6 viable cells; it is, of course, possible that one or more cells would be found even in the higher dilutions which would account for the growth recorded for the 10^{-9} mg/ml suspension.

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[6] Treffers, H. P., *Yale J. of Biol. and Med.*, 1946, **18**, 609.

When a medium is inoculated with increasing concentrations of cells, there is a corresponding decrease in the lag period of growth, but all of the resulting cultures finally reach the same degree of density. On the other hand, if the inoculum is held constant and the nutrient value of the medium is varied, quite different results are obtained. An increase in the amount of growth is found to parallel enrichment of the medium. This is accompanied by a shortening of the lag period.

The effect of nitrogenous materials on the rate of growth of *M. tuberculosis var. hominis* H37Rv is presented in Table II. The incubation time required for the initiation of growth in a medium containing ammonium citrate was 10 times that required in one containing casein, a vegetable extract and serum-albumin. The difference in the amounts of growth was even more striking, the more complex medium giving 20 times more abundant growth. The rate and amount of growth are dependent not only on the number and concentration of nutrients but on the nature of the nutritive material. Tryptophane in a concentration of 0.1% is a better nutrient than 0.2% asparagine for all of the strains of mycobacteria.

Having established the normal rate of growth of mycobacteria, it was now possible to evaluate the effect of streptomycin upon the growth of mycobacteria in the presence of different sources of energy. Regardless of the source of nitrogen or of the amount of growth supported by the medium, little difference was found in the amount of streptomycin required for bacteriostasis, as shown in Table III. The only exception is found in the ammonium citrate medium; limited effectiveness of streptomycin is possibly due to the high acidity of this medium resulting from the growth of the organism.[7] Similar results were obtained with *M. avium* and the human pathogenic varieties.

Summary. A simple, safe, and accurate method is described for the quantitative estimation of the growth of *Mycobacterium tuberculosis* based upon turbidimetric measurements in Dubos' medium.

This procedure can be applied to measuring the rate and amount of growth of *M. tuberculosis* in various media, as affected by the presence of streptomycin.

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[7] Waksman, S. A., Bugie, E., and Schatz, A., *Proc. Staff Meetings of Mayo Clinic*, 1944, **19**, 537.

15692

Preparation of an Anti-Ulcer Factor from Human Urine.*

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The preparation of crude extracts from urine (Urogastrone) and hog intestinal mucosa (Enterogastrone) which possess antigastric secretory and antiulcer activity in dogs, such as the Mann-Williamson and the Pavlov preparation has been reported in the literature. Two methods have been applied to urine for the preparation of Urogastrone or Urogastrone-like substances. Necheles[1] has prepared an inhibitor of gastric secretion and motility by treating normal human urine with ammonium sulphate and fractionating the resulting precipitate between vary-

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* The authors are indebted to Mr. and Mrs. Francis E. Fowler and members of their family whose generosity has made these studies possible.

[1] Necheles, H., Hanke, M. E., and Fantl, E., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 618.

ing concentrations of alcohol. Gray, Wiecyorowski and Ivy[2] used a benzoic acid adsorption method for the preparation of an antisecretory substance from normal male urine, and Friedman *et al.*[3] have found an inhibitory substance in normal female urine by using the same method of adsorption. An examination of the methods elicits little information regarding the chemical properties of the extracts except that they are water soluble and insoluble in organic solvents such as ethyl ether, benzene, and petroleum ether.[4] The further characterization of these extracts has been greatly retarded due to the lack of a convenient assay method.

The Mann-Williamson dog which has been generally used in testing for antiulcer properties of various preparations, entails considerable time, expense, and experience which greatly limits its usefulness. For an excellent discussion regarding the present status of these antiulcer "hormones" see the reviews of Ivy[5] and Sandweiss.[6]

With the recent availability of the rat assay method[7,8] in which extensive gastric ulceration can be produced in the rat in the short period of 7-9 hours following ligation of the pylorus, the investigation of antiulcer factors has been continued. A fraction has been prepared from human urine which when given to rats in sufficient quantity at the time of pyloric ligation prevents the formation of the gastric ulcers. The method consists essentially of adsorbing the activity on carbon from clarified urine at neutral pH, eluting the activity from the carbon with acidified aqueous-acetone at pH 2, and precipitating the activity from the eluate by increasing the acetone concentration. This method has been used continuously in this laboratory over a period of 6 months without obtaining an inactive preparation.

Experimental. The urine used in these studies was obtained over 24-hour periods from male and female patients who were in the clinic mainly for diagnostic purposes. Most of this urine was collected in the early morning hours and processed within 8 hours. The urine, which was collected in 2.5 liter bottles and preserved with chloroform, was decanted from the chloroform before carrying out the initial clarification with Celite-545.

The Assay procedure used in these experiments has been mentioned elsewhere.[8] The method consists of using male rats 8 weeks of age or female rats 10 weeks of age weighing 120 to 150 g. The animals are fasted for 48 hours before operation in individual cages with wide wire mesh bottoms, supported well above the dropping paper. Water is allowed *ad libitum.* For the operative procedure the rats are anesthetized with ether. Through a short midline incision extending downward from the xiphoid process, the duodenum is grasped lightly with ring forceps and a ligature of linen thread is placed exactly at the pyloric sphincter. The incision is closed with Michele wound clips and the wound is covered with a thin coating of flexible collodion. At the time of the operation the material to be tested for antiulcer activity is administered intravenously to the test group and physiological saline is given to the control group. A minimum of 6 to 10 rats should constitute a test group and an equal number is used for the control group. Eight or 9 hours after tying off the pylorus the animals are sacrificed. The abdominal cavity is opened, when the animal is under ether anesthesia, and the esophagus is grasped with a hemostat. The stomach is then freed of mesentery and removed. A small slit is made on the greater curvature of the distended stomach for collection of the gastric juice. The stomach is finally cut along the whole length of the greater curvature and after a brief rinsing in physiological saline, is stretched out with the aid of pins on beeswax for examination

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[2] Gray, J. S., Wieczorowski, E., and Ivy, A. C., *Science*, 1939, **89**, 489.

[3] Friedman, M. H. F., Recknayel, R. O., Sandweiss, D. J., and Patterson, T. L., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 509.

[4] Gray, J. S., Wieczorowski, E., Wells, J. A., and Harris, S. C., *Endocrinology*, 1942, **30**, 129.

[5] Ivy, A. C., *Fed. Proc.*, 1945, **4**, 222.

[6] Sandweiss, D. J., *Gastroenterology*, 1945, **5**, 404.

[7] Shay, H., Komarov, S. A., Fels, S. S., Meranze, D., Gruenstein, M., and Siplet, H., *Gastroenterology*, 1945, **5**, 43.

[8] Pauls, F., Wick, A. N., and MacKay, E. M., *Science*, 1946, **103**, 673.

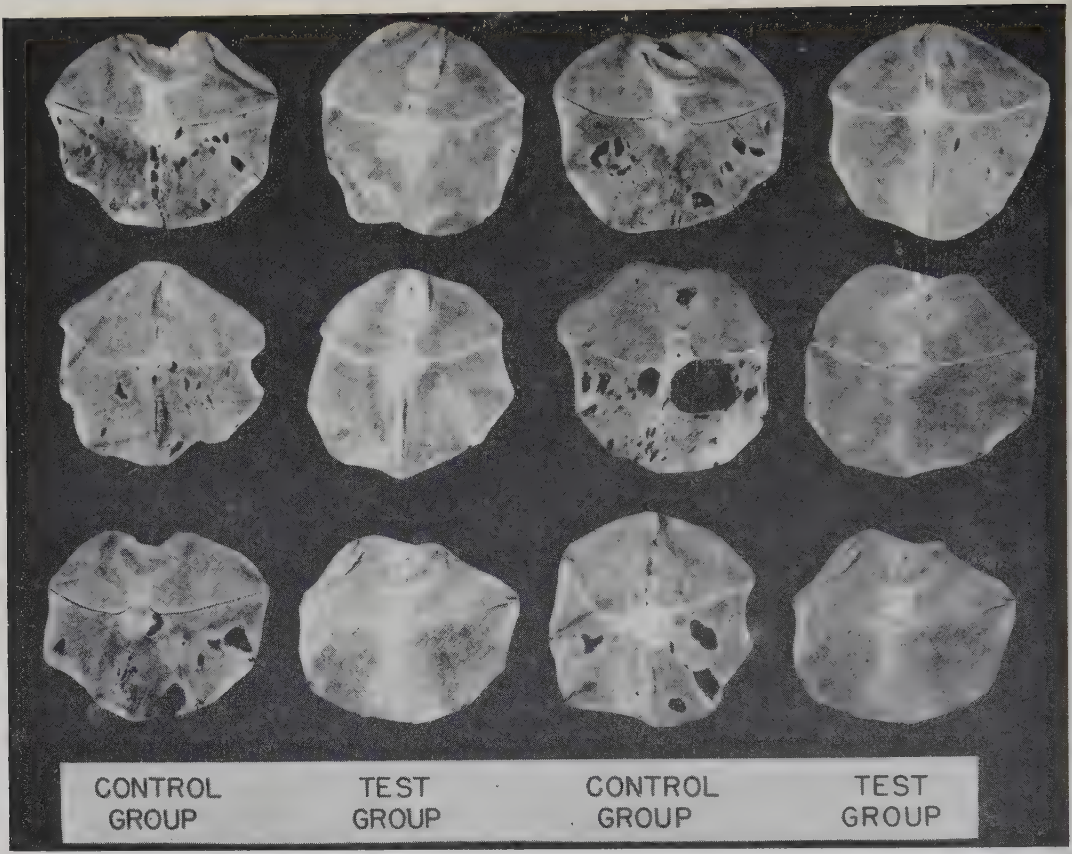


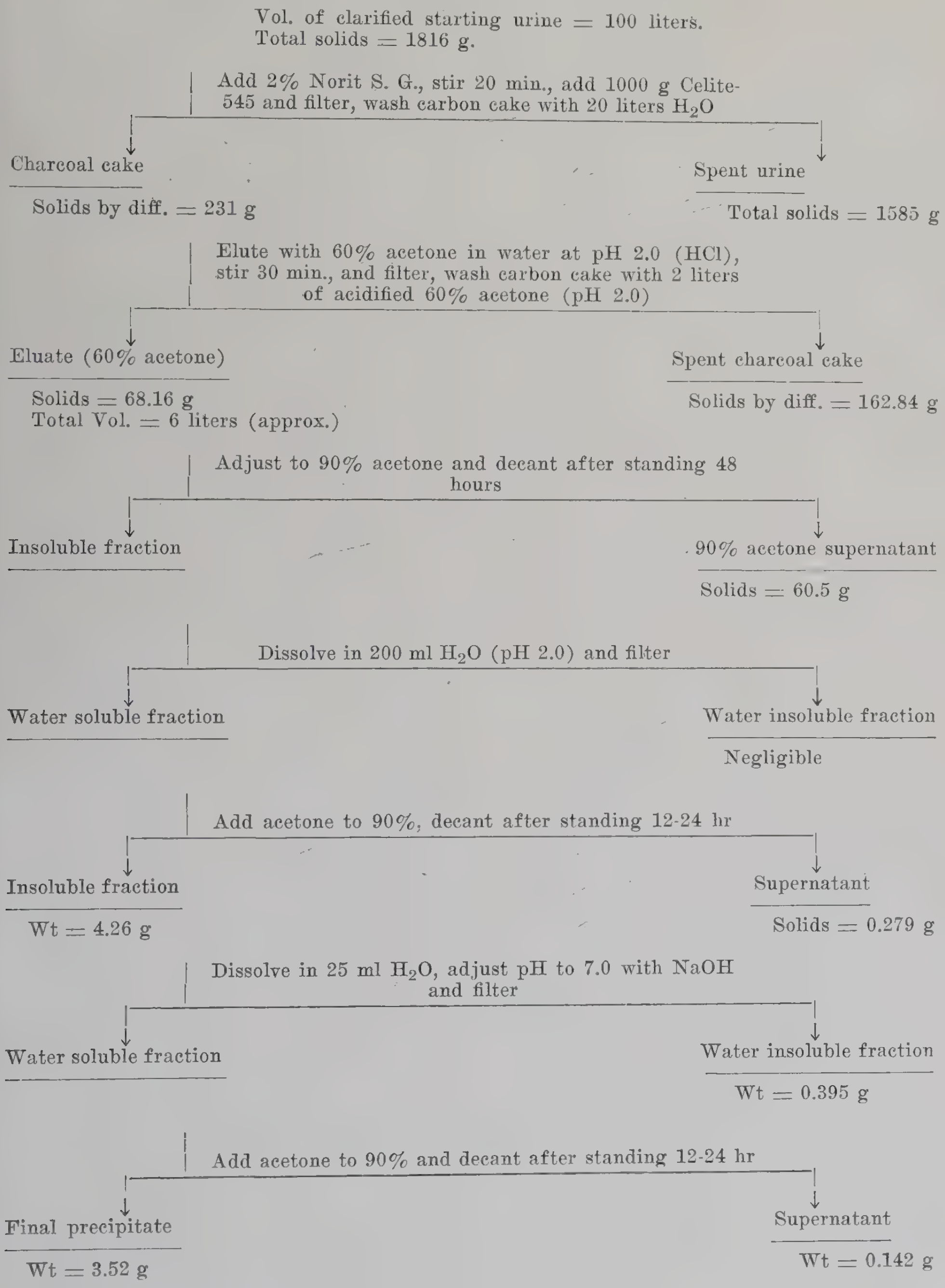
FIG. 1.

of the ulceration.

The steps for the preparation of the anti-ulcer substance are described for 100 liters of urine. Together with the distribution of solids they are outlined in detail in the accompanying flow sheet. The routine used in this laboratory is to pool all of the urine each morning and to clarify it by filtration with the aid of 1% Celite. The pH of the clarified urine has ranged from 6.0 to 6.5 and no further adjustment is made before the addition of the carbon. The percent Norite S.G. has been found to give satisfactory results for the adsorption of the activity. After the suspension is stirred for 20 minutes, the carbon is collected in large Buchner funnels. Celite is added to the mixture before filtering in order to facilitate the filtration procedure. The charcoal cakes thus obtained each day are stored in 4°C cold room until the end of the week at which time they are combined for the elution of the active substance. The elution is carried out in 60% acetone by volume in water at pH 2.0. The water content of the carbon cake, which is readily determined by weighing, is taken into account when adjusting to the 60% acetone concentration. Additional amounts of 60% acetone in water are added if necessary to prepare a thin slurry for efficient stirring. Although one elution is carried out in these experiments, a second and third elution can be used if so desired. The final product, after the neutralization and the subsequent filtration, can be obtained by the lyophile process or by increasing the acetone concentration as shown in the flow sheet.

Results. The preparation obtained in this carbon-acetone method will prevent complete-

FLOW SHEET FOR THE ISOLATION OF AN ANTI-ULCER EXTRACT FROM URINE.



Vol. of clarified starting urine = 100 liters.
Total solids = 1816 g.

Add 2% Norit S. G., stir 20 min., add 1000 g Celite-545 and filter, wash carbon cake with 20 liters H_2O

Charcoal cake
Solids by diff. = 231 g

Spent urine
Total solids = 1585 g

Elute with 60% acetone in water at pH 2.0 (HCl), stir 30 min., and filter, wash carbon cake with 2 liters of acidified 60% acetone (pH 2.0)

Eluate (60% acetone)
Solids = 68.16 g
Total Vol. = 6 liters (approx.)

Spent charcoal cake
Solids by diff. = 162.84 g

Adjust to 90% acetone and decant after standing 48 hours

Insoluble fraction

90% acetone supernatant
Solids = 60.5 g

Dissolve in 200 ml H_2O (pH 2.0) and filter

Water soluble fraction

Water insoluble fraction
Negligible

Add acetone to 90%, decant after standing 12-24 hr

Insoluble fraction
Wt = 4.26 g

Supernatant
Solids = 0.279 g

Dissolve in 25 ml H_2O, adjust pH to 7.0 with NaOH and filter

Water soluble fraction

Water insoluble fraction
Wt = 0.395 g

Add acetone to 90% and decant after standing 12-24 hr

Final precipitate
Wt = 3.52 g

Supernatant
Wt = 0.142 g

ly the formation of gastric ulcers in our rat-assay method. The quantity of material required to inhibit the ulceration has varied in different preparations from 50 to 100 mg/rat. Fig. 1 shows the stomachs removed from 6 control rats which received injections of physiological saline, and 6 test animals which received 50 mg of the urinary product intravenously at the time of pyloric ligation. The control group all show marked ulceration, while those in the test group receiving the antiulcer substance show a marked diminution or a complete absence of ulceration. Such clear cut results are not always obtained, especially when the minimal active dose of antiulcer material is being investigated. For such cases we have been recording the gastric ulceration by an arbitrary scale which ranges from "clear" or "zero" to "4 plus" ulceration.

Discussion. The chemical nature of the urinary antiulcer factor described here has not been determined, although the carbon-acetone method, which we have used for its preparation, is similar to that used for the isolation of the basic substance streptomycin.[9] A discussion of the chemical relationship between Urogastrone, Enterogastrone, and the urinary "carbon-acetone" material and proof that the antiulcer activity of the "carbon-acetone" produced extract is due to a single chemical entity must wait until further purification can be carried out.

Our urinary line product is approximately 5% ash and contains 5-6% nitrogen. The neutralized material is insoluble in organic solvents which are immiscible with water. The product obtained by acidifying a solution of the antiulcer factor to pH 2.0 and drying by the lyophile process is soluble to the extent of approximately 1 g per 100 ml methyl alcohol at room temperature. This fraction has greater activity than the starting material or the methyl alcohol insoluble fraction. The antiulcer activity of the crude line product can be more readily increased by fractionating with aqueous-acetone. In a 5% aqueous solution of starting material, the fraction precipitated by increasing the acetone concentration from 30 to 60% contains 30% of the solids and approximately 60% of the starting activity.

The antiulcer factor is fairly stable to alkaline hydrolysis. When 800 mg is dissolved in 20 ml of 0.1 N NaOH and refluxed for 24 hours, approximately 50% of the activity is retained. In contrast, when an equal quantity of the material is dissolved in 20 ml of 0.1 N HCl and refluxed for one hour, most if not all of the activity is destroyed. Shorter periods of acid hydrolysis have not been studied although the acid solution darkens rapidly after the start of refluxing.

We are unable at the present time to estimate the concentration of the antiulcer factor normally occurring in the urine. Assays carried out on the clarified and spent urine at maximum tolerable levels (200 mg/rat) have shown no reduction in gastric ulceration. The "90% acetone supernatant" which constitutes 85 to 95% of the solids in the "Eluate" has slight activity at 100 mg/rat. This fraction has an ash content of 28% and since we have observed that it is quite toxic at therapeutic doses, we are hesitant in attributing much importance to this fraction.

No attempt can be made at this time to speculate on the mechanism of the antiulcer effect exerted by the material prepared from urine. Ulceration may be reduced in extent or entirely prevented by a reduction in the secretion of gastric juice, impaired peptic activity of this juice or an enhancement of the resistance to ulceration which is inherent in the gastric mucosa.

Summary. A neutral extract has been prepared from normal human urine by adsorption on charcoal and elution thereof with 60% acetone in water at pH 2. This material, when given to rats at the time of pyloric ligation, prevents the development of the gastric ulceration which normally occurs with this procedure.

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[9] Vander Brook, M. I., Wick, A. N., De Vries, W. H., Harris, R., and Cartland, G. F., *J. Biol. Chem.*, 1946, **165**, 463.

15693

Antagonism of the Hemorrhagic Syndrome Induced by Derivatives of 3-Hydroxy-1,4-Naphthoquinone.*

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It has been reported recently[1] that certain 2-substituted-3-hydroxy-1,4-naphthoquinones, when administered to the white rat, induce a hemorrhagic syndrome which is associated with a hypoprothrombinemia. Preliminary studies showed that the hemorrhagic activities of at least one of these naphthoquinones, 2-(3-cyclohexylpropyl)-3-hydroxy - 1,4-naphthoquinone (SN 5090†), could be antagonized completely by simultaneous administration of vitamin K_1. Similar administration of 2-methyl-1,4-naphthoquinone had less marked effect. Subsequent to this work experiments were carried out on 2 other 2-substituted-3-hydroxy derivatives. When it was found that these compounds, like the naphthoquinones described previously,[1] also induced a hemorrhagic syndrome and hypoprothrombinemia it was decided to expand the previous observations on the antagonistic action of vitamin K_1 and 2-methyl-1,4-naphthoquinone. The present report deals primarily with this phase of the study.

Experimental. The compounds studied included 2-cyclohexyl-3-hydroxy-1,4-naphthoquinone (SN 5094), 2-(2-methyloctyl)-3-hydroxy-1,4-naphthoquinone (SN 5949), and 2-[3 - (*p*-phenoxyphenyl)propyl]-3-hydroxy-1,4-naphthoquinone (SN 13,936‡). All 3 of these naphthoquinones induced a hemorrhagic syndrome characterized by gross extravasations of blood into the subcutaneous tissues of the limbs, head, neck and scrotum, and diffuse bleeding of the gastrointestinal mucosa; associated with this syndrome were greatly prolonged clotting and prothrombin times. SN 5094 was the least toxic of the compounds; daily doses of 400 mg per kg uniformly produced fatal lesions but doses of 200 mg had little effect. SN 5949 was somewhat more toxic; it usually produced fatal lesions at 200 mg per kg but had little effect at lower doses. SN 13,936 was the most toxic of the compounds; it uniformly was fatal at 200 mg per kg doses and produced severe hemorrhages at doses of 100 mg.

The technics employed in studying the antagonistic effects of vitamin K_1 and 2-methyl-1,4-naphthoquinone were similar to those used in the previous study.[1] Male rats, 4 to 5 weeks old, Sprague-Dawley strain, were used throughout and were fed a diet of Purina Dog Chow checkers *ad libitum.*

The drugs and vitamin adjuncts were suspended or dissolved in olive oil and were administered once daily via stomach tube. Treatments were continued for 8 to 12 days where the rats survived that long. Whenever it was possible to obtain blood samples, prothrombin times were measured. Determinations were carried out on whole blood using the method of Kato[2] with Russell viper venom as the source of thromboplastin. The results of the pertinent experiments have been summarized in Table I.

In the experiments with SN 5094, daily doses of vitamin K_1 of 10 mg per kg completely prevented the development of the hemorrhagic syndrome. Similar doses of 2-methyl-1,4-naphthoquinone were somewhat less effective. Hemorrhagic lesions appeared on the 4th day of the experiment but appeared to regress with continued treatment. At the end of the experiment normal pro-

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* The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Institute of Medical Research, The Christ Hospital, Cincinnati, Ohio.

[1] Smith, Carl C., Fradkin, R., and Lackey, M. D., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 398.

† The numbers cited here are those assigned to the compounds by the Survey of Antimalarial Drugs, Baltimore, Md.

‡ The drugs were prepared by Dr. Louis F. Fieser, Harvard University, and the Abbott Laboratories, to whom we are greatly indebted.

[2] Kato, K., *Am. J. Clin. Path.*, 1940, **10**, 147.

TABLE I.
The Effectiveness of 2-Methyl-1,4-Naphthoquinone and Vitamin K_1 on the Hemorrhagic Activities of SN 5094, SN 5949, and SN 13,956.

			Effects of treatment	
Rat No.	Daily treatment, mg/kg body wt	Days treatment	Prothrombin time, sec	
1	400 mg SN 5094	4	—	Died 4th day; typical hemorrhagic lesions.
2		4	—	" " " " " "
3		5	—	" 5th " " " "
4		6	—	" 6th " " " "
5		8	—	" 8th " " " "
6	400 mg SN 5094 and 10 mg 2-methyl-1,4-naphthoquinone	6	—	" 6th " " " "
7		11	30	Hemorrhagic lesions on limbs.
8		11	23	" " " "
9		11	17	Regressing hemorrhagic lesion in scrotum.
10	400 mg SN 5094 + 10 mg vit. K_1	11	20	No hemorrhagic lesions.
11		11	18	" " "
12		11	17	" " "
13		11	14	" " "
14	200 mg SN 5949	3	—	Died 3d day; no hemorrhagic lesions.
15		5	25*	Hemorrhagic lesions on limbs and head.
16		8	190	" " " " " scrotum.
17		8	50	No hemorrhagic lesions.
18	200 mg SN 5949 and 10 mg 2-methyl-1,4-naphthoquinone	4	—	Died 4th day; no hemorrhagic lesions.
19		8	35	Regressing hemorrhagic lesions on limbs.
20		8	30	No hemorrhagic lesions.
21		8	25	" " "
22	200 mg SN 5949 and 10 mg vit. K_1	8	15	" " "
23		8	12	" " "
24		8	11	" " "
25		8	10	" " "
26	200 mg SN 13,936	3	—	Died 3rd day; gastrointestinal hemorrhages.
27		4	—	" 4th " typical hemorrhagic lesions.
28		5	>600	" 5th " " " "
29		6	—	" 6th " " " "
30		8	>600	" 8th " " " "
31	200 mg SN 13,936 and 10 mg 2-methyl-1,4-naphthoquinone	3	—	" 3rd " gastrointestinal hemorrhages.
32		6	—	" 6th " typical hemorrhagic lesions.
33		9	—	" 9th " " " "
34		10	—	" 10th " " " "
35	200 mg SN 13,936 and 10 mg vit. K_1	11	30	No hemorrhagic lesions.
36		11	40	" " "
37		11	60	" " "
38		11	140	" " "
39	200 mg SN 13,936 and 100 mg vit. K_1	12	12	" " "
40		12	14	" " "
41		12	15	" " "
42		12	18	" " "

* Prothrombin time obtained on 7th day. Animal too ill for treatment after 5th day.

thrombin times were obtained.

The findings with SN 5949 were similar to those just described. Vitamin K_1 in 10 mg per kg doses prevented the development of hemorrhagic lesions and hypoprothrombinemia whereas only a partial effect was obtained with this dosage of 2-methyl-1,4-naphthoquinone.

In the experiments with SN 13,936, 10 mg doses of 2-methyl-1,4-naphthoquinone were without apparent effect on the syndrome. Even vitamin K_1 at 10 mg per kg doses did not completely block the action of SN 13,936 for, although hemorrhagic lesions were absent, a significant hypoprothrombinemia was present. At 100 mg per kg doses, however, vitamin K_1 blocked development of both the hemorrhagic lesions and the hypoprothrombinemia.

Discussion. The data presented above demonstrate that, as in the case of SN 5090,[†] vitamin K_1 and to a lesser extent 2-methyl-1,4-naphthoquinone antagonize the hemorrhagic syndrome and hypoprothrombinemia induced by SN 5094, SN 5949 and SN 13,936. This finding would seem to strengthen the suggestion that the 2-substituted-3-hydroxy-naphthoquinones exert their hemorrhagic activity by competing with the K vitamins in whatever processes these substances serve in prothrombin formation.

This suggestion appears to offer the only tenable explanation for the hemorrhagic activity of the above naphthoquinones. The compounds do not produce severe liver injury, nor are they especially active antibacterial agents which might interfere with the synthesis of K vitamins by intestinal bacteria. Furthermore, extensive *in vitro* experiments indicate that they do not inactivate prothrombin as do the indandiones.[3] It appears, therefore, as if the relationship of the 2-substituted-3-hydroxy-1,4-naphthoquinones to vitamin K affords another example of antagonism of metabolites by structurally related compounds.

Summary. The development of the hypoprothrombinemia and hemorrhagic lesions, which occurred in the albino rat as the result of ingestion of various 2-substituted-3-hydroxy-1,4-naphthoquinones, could be prevented either partially or completely by simultaneous administration of 2-methyl-1,4-naphthoquinone or vitamin K_1. The amounts of these K vitamins required to block development of the above syndrome differed with the various hydroxy-naphthoquinone derivatives. In each instance, however, vitamin K_1 proved more effective than 2-methyl-1,4-naphthoquinone.

Observations made in this report confirm our earlier suggestion that the hemorrhagic syndrome, which results from administration of 2 - substituted - 3 - hydroxy-1,4-naphthoquinones, is due to competition between these substances and the K vitamins in processes involved in prothrombin formation.

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[3] Kabat, H., Stohlman, E. F., and Smith, M. I., *J. Pharm. and Exp. Therap.*, 1944, **80**, 160.

15694

Action of 3,3′ Methylenebis (4-Hydroxycoumarin) (Dicumarol) on Thromboplastic Activity of Rabbit Brain.*

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Several observations suggest that other factors besides the interference with the formation of prothrombin[1] must be considered in the pathogenesis of the bleeding tendency produced by dicumarol. Widespread dilatation of capillaries, arterioles and venules has been found, indicating that the hemorrhagic condition is due, at least in part,

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* This research was supported by a grant from the United States Public Health Service.

† Assistant, Medical Clinic of the University of Rome; at present, Research Fellow, Institute of International Education.

‡ Research Fellow, Department of Biochemistry, School of Medicine, Marquette University, Milwaukee, Wisc.

[1] Quick, A. J., *The Hemorrhagic Diseases*, pp. 284-285, C. C. Thomas, Springfield, Ill., 1942.

TABLE I.

Influence of Dicumarol Poisoning on Thromboplastic Activity of Brain Tissue (Rabbit) When Tested Against Normal Plasmas.

Thromboplastin preparation from Rabbit No.	Prothrombin time (in sec)														
	Human plasma					Rabbit plasma					Dog plasma				
	N.*	1†	2†	6†	8†	N.	1	2	6	8	N.	1	2	6	8
$CaCl_2$ concentrations															
.02 Molar	12	14	12½	13½	14	6	7	10½	9	9	6	11	8	8½	8
.01 ,,	11½	12½	12½	12½	13½	6	7½	11	9	9	6	11	8½	9	8
.005 ,,	11½	12½	12½	12	14	6	8	11	9½	9½	6	11	9½	9½	8½
.0025 ,,	11	13½	13½	11½	15	5½	9	11	10	10½	5½	11	9	10	9
.00125 ,,	14½	19½	20	16	22½	6	13	12	12	12½	6	11	9½	12	10
.0006 ,,	26	72	39	48	43	8½	20	24	19	18	8½	14	13	17	16
.0005 ,,	42	115	69	127	75	10½	28	31	22	27	12	19	22	21	20

* N.: Normal control.

	Prothrombin time at time of death using 0.01 M $CaCl_2$
† Rabbit 1: Killed on 4th day (dicumarol by stomach tube)	28.5 sec.
,, 2: ,, ,, 7th ,, ,, ,, ,, ,,	∞
,, 6: ,, ,, 7th ,, ,, intravenously)	∞
,, 8: ,, ,, 7th ,, ,, ,,	92 min.

to vascular damage.[2] Moreover, other factors of the normal mechanism of coagulation have been found to be affected by dicumarol. Adhesiveness of the platelets is decreased[3,4] and the minimum requirement of calcium for optimum prothrombin time is increased[5,6] during the administration of the drug.

Evidence has been obtained which indicates that dicumarol causes changes in the thromboplastic activity of tissues. Dyckerhoff[7] claims that dicumarol inhibits the first phase of coagulation by depressing the activity of thrombokinase. Recently Bose[8] has shown that thromboplastin prepared from desiccated brain of rabbits which have been fed 10 mg of dicumarol for 3 days is less active than that prepared from normal brains when tested on normal rabbit plasma using the prothrombin time technic.

In the present report, thromboplastic activity of rabbit brain obtained from animals severely poisoned by dicumarol has been studied by the prothrombin time method of Quick, but using a standard series of dilutions of $CaCl_2$. Plasma from men and from normal and dicumarol-treated dogs and rabbits was employed.

Experimental. Ten full grown male rabbits in good state of nutrition were given dicumarol [3,3′-methylenbis(4-hydroxycoumarin)] until the prothrombin level was so depressed that they either died from hemorrhage or their prothrombin time and coagulation time were so delayed that neither one could any longer be determined satisfactorily. The drug was administered in doses of 5 mg per kg of body weight per day. In some animals the drug was administered orally as a gum acacia suspension by stom-

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[2] Bingham, J. B., Meyer, O. O., and Pohle, F. J., *Am. J. Med. Sc.*, 1941, **202**, 563.

[3] Spooner, M., and Meyer, O. O., *Am. J. Physiol.*, 1944, **142**, 299.

[4] Wright, H. P., *J. Path. Bact.*, 1945, **57**, 382.

[5] Jaques, L. B., and Dunlop, A. P., *Am. J. Physiol.*, 1945, **143**, 355.

[6] Quick, A. J., *Am. J. Physiol.*, 1947, **148**, 211.

[7] Dyckerhoff, H., *Bioch. Z.*, 1944, **316**, 397, (*Chem. Abstracts*, 1945, p. 551).

[8] Bose, A. N., *Ann. Biochem. Exp. Med.*, 1945, **5**, 105.

TABLE II.

Influence of Dicumarol Poisoning on Thromboplastic Activity of Brain Tissue (Rabbit) When Tested Against Plasmas of Animals Treated with Dicumarol (Single Intravenous Dose of 5 mg per Kilo Weight).

	Prothrombin time (in sec.)																	
	Dog* plasma															Rabbit plasma		
Days after injection	2		6			7		9		8			15			1		
Animal No.	5		5			2		2		6			6			8		
Thromboplastin preparation from Rabbit No.	N.†	3‡	N.	3	4	N.	1	N.	3	N.	5	6	N.	7	9	N.	5	6
$CaCl_2$ concentrations																		
.02 Molar	18	24	10	13	13½	7	11½	7	9½	20½	37½	36	8½	12	13	9	13	14
.01 ,,	18½	25	10½	12½	13½	7	11	7	10	20	43	48	8	10½	12	9	13	13
.005 ,,	19	26	10½	14	14	6½	11	7	10	24	53	54	8½	11	12	9	13	14
.0025 ,,	23	31	11	15	15	6½	11	6½	10	26	59	68	9	11	12	10½	14	15
.00125 ,,	45	52	12	24	16	6	12½	6	11½	59	121	166	9	13	14	13	22	22
.0006 ,,	132	207	17	50	43	11	30	10	28	69	184	192	13	17	19	42	94	103
.0005 ,,	—	—	20	67	55	12	35	—	—	—	—	—	17	20	27	45	141	192

* The above are representative figures from 7 dogs.

† Normal control.

	Prothrombin time at time of death using .01 M $CaCl_2$
‡ Rabbit 3: Killed 7th day (dicumarol by stomach tube)	∞
,, 4: ,, 6th ,, ,, intravenously)	102 min.
,, 5: ,, 7th ,, ,, ,,	20 ,,
,, 7: Died 7th ,, ,, ,,	∞
,, 9: Killed 7th ,, ,, stomach tube)	108 ,,

ach tube; in others intravenously as the sodium salt and in a third group both the oral and the intravenous routes were used. Thromboplastin was prepared by the acetone dehydration procedure of Quick.[9] One-tenth of 0.1 M. sodium oxalate per rabbit brain was added before triturating with acetone. By this means any calcium in the brain tissue was removed, since no detectable amount of this element was found by ordinarily chemical methods in 0.2 g of the dried rabbit brain powder. The prothrombin time of oxalated human, dog and rabbit plasma was determined by the procedure of Quick. A standard series of dilutions of $CaCl_2$ solutions was used since the lower concentrations have been shown to be more sensitive to minimum changes of the prothrombin time.

Results and Discussion. It is evident from the results in Table I that the thromboplastin prepared from the brains of rabbits poisoned with dicumarol was less active than that obtained from normal animals. With dogs' and rabbits' plasmas this difference is obtained with the various dilutions of calcium used but becomes more accentuated in the lower concentrations. With human plasma this difference is obtained only in the lower concentrations.

In comparing the thromboplastin from dicumarolized rabbits with that of normal animals on rabbit and dog plasmas in which the prothrombin was depressed by dicumarol, the difference in activity was found to have a wider spread (Table II) but followed essentially the pattern of normal oxalated plasma in regard to the influence of calcium. Occasionally when the prothrombin is presumably normal as measured with fully active thromboplastin, a distinct delay is brought out by dicumarol thromboplastin as shown in the case of dog No. 2 on the 9th day after a single dose of dicumarol intravenously.

It is somewhat surprising that dicumarol should decrease the activity of the thromboplastin in brain tissue and this naturally leads to the question whether the thromboplastin occurring in blood is similarly affected. If this be the case, then the delayed coagulability due to dicumarol would have a 3-fold cause: decrease of prothrombin level, inadequate calcium for optimum prothrombin activity and less active thromboplastin.

What importance the latter factor has is difficult to evaluate especially since the diminution in thromboplastic activity is not particularly marked. In view of the fact that the nature of thromboplastin is poorly understood and the action of dicumarol is not completely known, it would be futile to attempt to offer any possible explanation for the results presented in this paper.

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[9] Quick, A. J., *Science*, 1940, **92**, 113.

15695

Grisein, a New Antibiotic Produced by a Strain of *Streptomyces griseus*.*†

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Streptomyces griseus represents a large and heterogeneous group of actinomycetes, widely distributed in nature. Cultures belonging to this species or species-group have been isolated from a great variety of substrates, largely soils, peats, composts and animal contents. Since the first report[1] on the production of streptomycin by 2 cultures of *S. griseus* obtained from 2 different substrates,

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* Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology.

† Partly supported by a grant made by the Commonwealth Fund of New York.

[1] Schatz, A., Bugie, E., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 66.

TABLE I.
Spectrum of Cultures of *S. griseus* Grown in Nutrient and Corn Steep Broths.
Dilution units per 1 milliliter.

	Nutrient broth	Corn steep broth
Escherichia coli	250	30
Aerobacter aerogenes	0	0
Serratia marcescens	250	50
Pseudomonas aeruginosa	5	0
” *fluorescens*	0	0
Proteus vulgaris	0	0
Mycobacterium phlei	0	0
Bacillus subtilis	200	30
” *mycoides*	0	0
” *megatherium*	100	100
Staphylococcus aureus	200	200
Sarcina lutea	0	0

nearly 100 cultures of this organism or closely related forms have been isolated.[2] However, very few of these were found to be capable of producing streptomycin. Actually, one other culture among the many isolated in our laboratory had the capacity of forming authentic streptomycin.[3] Two other streptomycin-producing strains were reported from another laboratory.[4]

Morphologically, *S. griseus* is characterized by the formation of clusters or tufts of sporulating hyphae in the aerial mycelium. The most characteristic cultural property is the production on suitable media of an abundant, powdery, greyish-green aerial mycelium. No distinct chromogenic (dark brown to black) pigment is produced on organic media; occasionally a greenish to light brownish pigment is observed just below the vegetative growth. The typical cultures may give rise to variants which differ from the original organism both in their morphology and in their physiology. Two variants have already been isolated, one of which is devoid of aerial mycelium[5] and the other showing certain other cultural characteristics which are distinct from those of the mother culture; both variants are unable to produce streptomycin. The ability of other variants to produce streptomycin may be greatly reduced.

For the isolation of *S. griseus* from natural substrates, media enriched with streptomycin have been utilized.[3] Such media prevent the development of most of the bacteria and the great majority of actinomycetes. Among a large number of cultures isolated by this method, one was found to form an antibiotic which at first appeared to be streptomycin-like in nature. This culture (G-25) was isolated from a sample of Huleh peat, obtained from Palestine. The antibiotic was produced on a variety of media, of which peptone-meat extract and starch-tryptone had the highest activity. Culture filtrates of the organism were found to inhibit the growth of various Gram-negative bacteria, notably *Escherichia coli,* as well as certain Gram-positive bacteria, such as *Bacillus subtilis* and *Staphylococcus aureus.*

In spite of certain apparent similarities of this antibiotic to streptomycin and to streptothricin, 4 important differences were noted, suggesting that one is dealing here with a type of substance distinct from the others: 1. The bacteriostatic spectrum of the new agent was much narrower than that of either streptomycin or streptothricin. A typical culture filtrate of G-25 had a titer of 300 *E. coli* and 300 *B. subtilis* dilution units, but,

TABLE II.
Bacteriostatic Spectra of Grisein and Streptomycin.
Units per gram of crude preparations.

	Grisein × 1,000	Streptomycin × 1,000
Escherichia coli W*	25	25
Serratia marcescens	10	25
Proteus vulgaris	<.1	10
Pseudomonas fluorescens	3	2
Ps. aeruginosa	<.1	1
Aerobacter aerogenes	<.1	10
Salmonella schottmülleri	10	15
” *aertryke*	<.1	3
Eberthella typhi	<.1	25
Shigella sp.	30	25
Klebsiella pneumoniae	5	25
Mycobacterium phlei	<.1	100
Bacillus subtilis	10 to 30	125
” *megatherium*	10 to 20	100
” *mycoides*	<.1	20
” *cereus*	<.1	30
Staphylococcus aureus	30 to 100	15
S. lutea	0.5	100
Micrococcus lysodeikticus	200 to 300	150

* Considerable variation in sensitivity of different strains has been observed.

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[2] Waksman, S. A., Schatz, A., and Reynolds, D. M., *Ann. N. Y. Acad. Sci.*, 1946, **48**, 73.

[3] Waksman, S. A., Reilly, H. C., and Johnstone, D. B., *J. Bact.*, 1946, **52**, 393.

[4] Carvajal, F., *Mycologia*, 1946, **38**, 596.

[5] Waksman, S. A., Reilly, H. C., and Schatz, A., *Proc. Nat. Acad. Sci.*, 1945, **31**, 157.

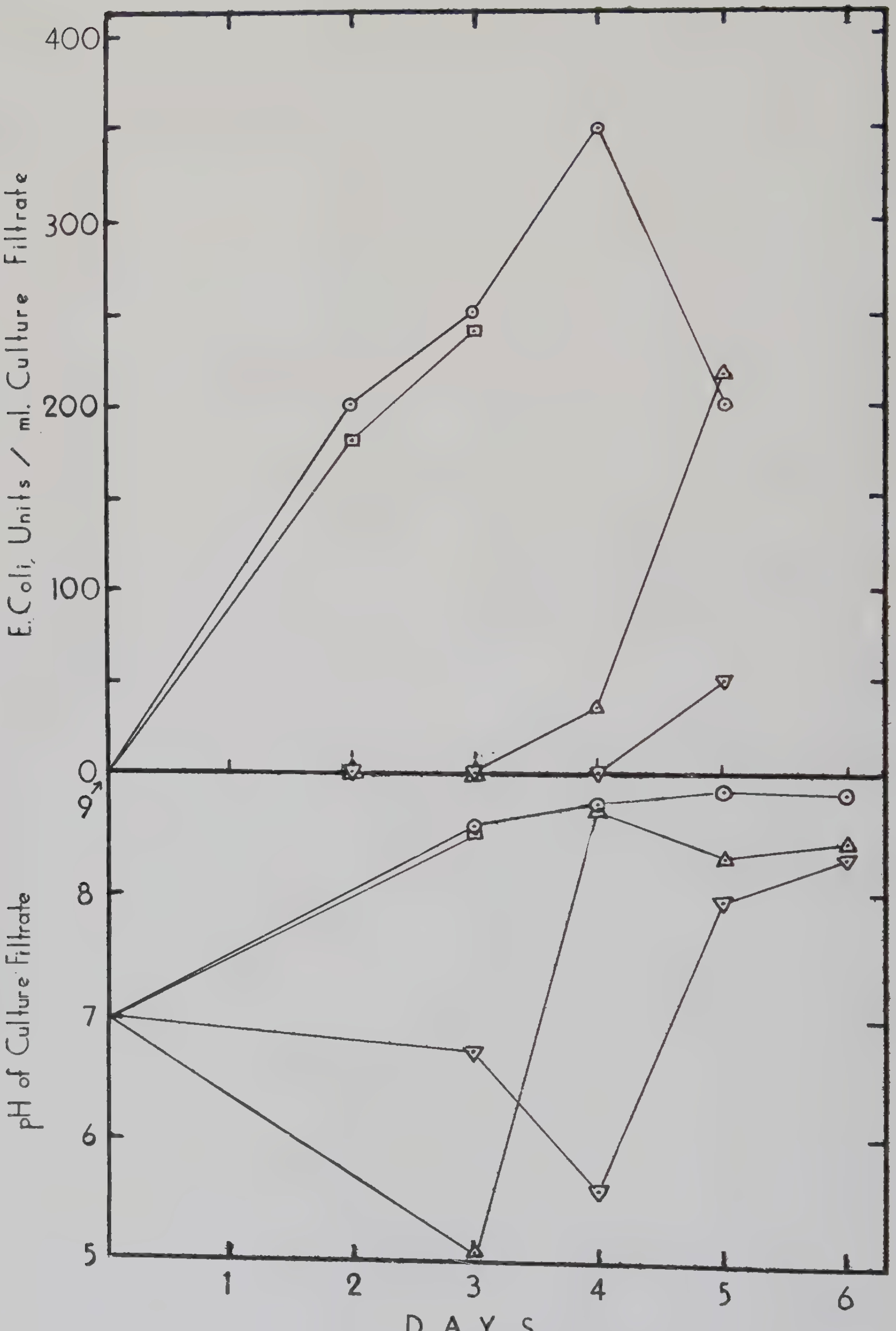

FIG. 1.

The Effect of Various Supplements on the Production of Grisein.

○ Basal medium—5 g peptone, 5 g NaCl, 10 ml neutralized corn steep liquor per liter. Supplements added—10 g/lit.; △, glucose; inverted △, glycerol; □, lactose.

unlike streptomycin, had no activity against either *B. mycoides* or *Aerobacter aerogenes.* Likewise, it had no activity against fungi, a characteristic which distinguishes it from streptothricin. 2. The chemical nature of the antibiotic produced by G-25 is different from that of the other 2 antibiotics, since, while readily adsorbed on charcoal, it could not be removed with acid alcohol. 3. Glucose did not bring about the inactivation of this antibiotic, though it usually inactivates both streptothricin and streptomycin. 4. Streptomycin-resistant strains of *E. coli* remained sensitive to this antibiotic.

Culture G-25 was grown both in stationary and in submerged cultures. The submerged culture underwent rapid, even if incomplete, lysis in 3 to 5 days. Media containing meat extract or corn steep, but free from glucose, gave the best activity. The bacteriostatic spectrum of a typical culture filtrate of this organism is brought out in Table I. The agar streak method[6] was used. The results point to a very narrow antibiotic spectrum, which is not limited either to the Gram-positive or to the Gram-negative bacteria (Table II).

When the culture filtrate was treated with activated charcoal (5 g per liter), all the antibiotic was removed. On treatment of the charcoal with 9% ethanol, about half of the total activity was recovered. The alcohol eluate was concentrated to a syrup *in vacuo,* and absolute methanol was added until a precipitate began to form. The final preparation was precipitated with acetone, washed with ether, and desiccated. A yield of 400-600 mg was obtained per liter of culture. This preparation showed an activity of about 15,000-30,000 *E. coli* dilution units per gram. More active preparations have often been obtained, ranging up to 100,000 *E. coli* units per gram. Unfortunately, only a small part, usually about 20%, of the total active substance present in the culture was thus recovered. The antibiotic spectrum of the isolated fraction was, however, exactly the same as that of the crude culture filtrate, thus pointing to the identity of the substance in the medium and of the isolated preparation.

It is proposed to designate this antibiotic substance as *grisein.* It is insoluble in ether, chloroform, absolute acetone, absolute ethanol, or benzene. It is slightly soluble in ordinary acetone and in 95% ethanol. It is readily soluble in water. It is heat-stable, since heating for 10 minutes at 100°C does not reduce greatly its activity. It is not affected if kept for short periods at pH levels ranging from 4.0 to 10.5.

The antibiotic activity of grisein can be measured by the dilution agar-streak and cup assay methods. With the cup method, *B. subtilis, S. aureus* and *E. coli* can be used as test organisms; the zone of inhibition, for the first organism, is usually narrower than that for comparable concentrations of either streptomycin or streptothricin, but it is wider and more distinct for the second and third organisms. The broth dilution method cannot be used very effectively for the study of grisein, since there are always a few cells in the culture of the test bacterium that are originally resistant or become readily resistant to this antibiotic; these cells begin to multiply rapidly, making impossible accurate readings by the dilution method. The rapid development of resistant strains from originally sensitive cultures makes studies of the bactericidal action of grisein upon bacteria in broth cultures rather difficult.

The presence of glucose or glycerol in the culture medium for the production of grisein results in very low yields of this antibiotic due to a lowering of the pH. However, as soon as the pH begins to rise, the antibiotic is formed, as shown in Fig. 1. Lactose, however, has no such effect.

Samples of crude grisein preparations were tested in mice infected with *Salmonella schottmülleri* and *S. aureus.* A single dose of 800 units (dilution units against *E. coli*) per mouse, administered subcutaneously immediately after infection, protected 100% of the animals infected with the first, and 1,600 units/mouse gave complete protection against the second. The antibiotic showed only little toxicity to experimental animals and was excreted readily and in an active state in the urine. Because of its low toxicity, its activity *in vivo* and also its activity upon streptomycin-resistant strains point to the possibility of utilizing grisein for the control of infections caused by bacteria resistant to other antibiotics. However, its narrow antibiotic spectrum would limit its practical application.

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[6] Waksman, S. A., and Reilly, H. C., *Anal. Ed., Ind. Eng. Chem.*, 1945, **17**, 556.

Summary. Streptomyces griseus is widely distributed in soils, peats and in composts. Only very few strains of this organism are capable of producing streptomycin. Most of the strains produce no antibiotic at all, whereas certain strains produce antibiotics that are distinctly different from streptomycin. One such new antibiotic was isolated and designated as grisein.

Grisein is active against certain Gram-positive and Gram-negative bacteria. Its antibacterial spectrum is much more limited than that of either streptomycin or streptothricin. Cultures of bacteria that are made resistant, by serial passage, to streptomycin still remain sensitive to grisein. Cultures of bacteria originally sensitive to grisein give rise easily to strains resistant to this antibiotic.

Grisein shows a rather low toxicity to experimental animals and is rapidly excreted in the urine. It was found capable of protecting experimental animals against infections with *S. schottmülleri* and *S. aureus*.‡

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‡ The authors are indebted to Mr. Otto Graessle, of the Merck Institute, for testing the animal toxicity of grisein, and to Dr. H. Boyd Woodruff, of the Merck Laboratories, for checking the sensitivity of streptomycin-resistant strains of different bacteria.

15696

Determination of Oxygen Consumption in the Albino Rat.*

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Technical aspects of the measurement of gaseous metabolism in the albino rat would appear, from a cursory survey of the literature, to be almost a closed chapter in biologic technology. Yet the determinations, as executed, have proved inaccurate, inconsistent, and time-consuming. The method to be described below appears to offer several distinct advances over those currently in use.

The machine devised for these studies is of the closed-circuit type, measuring only oxygen consumption. Its major structural features are illustrated in Fig. 1.

Animal chamber, spirometer box, and water bath are all constructed of heavy sheet brass, which is adequate for the rapid equalization of temperature throughout the system. The temperature is maintained within narrow limits by means of a tubular model heating element controlled through a suitable relay by a thermoregulator immersed in the water bath. The water is kept circulating by a noiseless electric stirrer. Sensitivity of the thermoregulator is assayed by a blank test, measuring the variations in volume of the inclosed oxygen that are induced by the automatic activation and deactivation of the heating element; such variations have not exceeded 0.5 cc. The temperature within the animal chamber is maintained at 28°C, stated by Benedict and MacLeod[1] to be the temperature of thermic neutrality for the albino rat.

Dimensions of the animal chamber are 9″ x 6″ x 5½″. Its lid is provided with a large window, composed of 2 panes of glass separated by an air space to limit condensa-

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* Paper No. 831, Research Series, the University of Arkansas.

[1] Benedict, F. G., and MacLeod, G., *J. Nutrition*, 1929, **1**, 367.

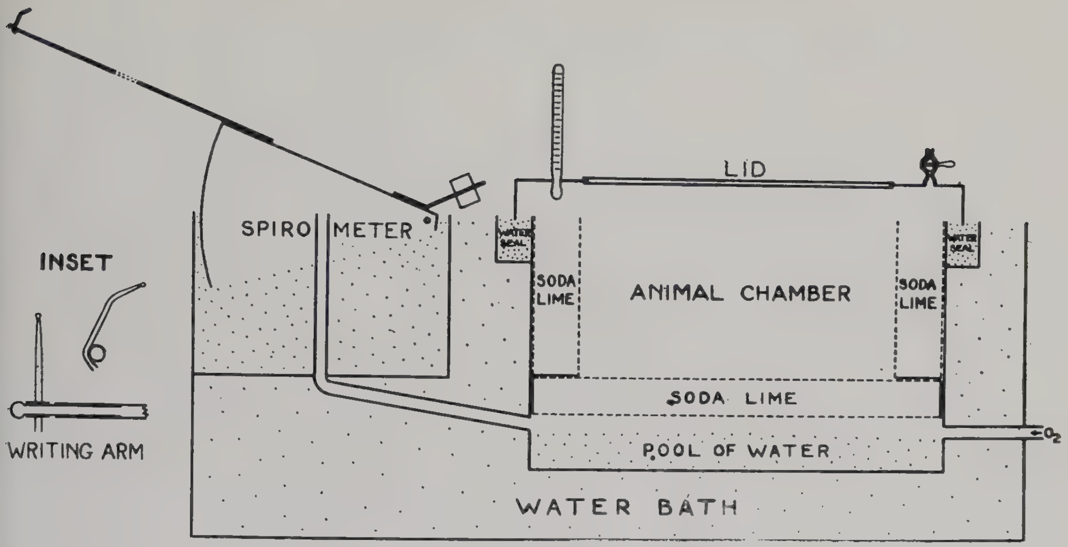

Fig. 1.
Basal Metabolism Machine. Longitudinal section. About ¼ scale. Heating element, thermoregulator, and water stirrer not shown. Inset: writing arm, side view as inserted into writing lever, end view of writing arm and collar without the pin which inserts into writing lever.

tion of water. The thermometer[†] and a stopcock, to allow free movement of air into the system whenever the apparatus is opened, are fitted into the lid. The chamber is rendered airtight by a water-filled moat into which the overhanging rim of the lid dips.

The rat is placed into a wire mesh cage, somewhat smaller than the space available, and this is introduced in turn into the animal chamber, where it is flanked fore and aft by soda lime boxes made of copper window screening. The inner cage rests on a false-bottom floor, formed by a third soda lime container, which is itself elevated about an inch above the true floor of the animal chamber in order to provide space for a pool of water below the CO_2 absorbent.

Koehler[2] points out the advisability of maintaining a relatively high humidity within the chamber, partly to keep the soda lime at maximum absorptive efficiency and partly to induce the animal to relax. He considers, however, that a humidity above 75% causes restlessness. The present author, on the other hand, observed that the soporific influence varies directly with the vapor tension and that the 99% humidity provided at 28°C by a pool of water is most effective, at least in the rat. Under that regime, the animal almost immediately composes itself for sleep, sometimes on the first trial, usually after 3 or 4.

The spirometer bell is made of thin-walled brush copper, which is far less vulnerable than aluminum to attack by soda lime. It is a Krogh type spirometer, pivoting on jewel bearings and thus providing freedom of movement without the lateral shifting encountered in the ordinary cylindrical spirometer. The bell is counterweighted in such manner that, with the system open to the outside, it descends slowly and without acceleration. Its dimensions are 5″ x 2½″ x ½-3½″, giving a volume of about 300 cc, which is well above the requirement of even a hyperthyroid rat. Customarily, however, the spirometer is refilled during the rest period about 10 minutes before the test run; this is done from an ordinary vitalometer in order to avoid disturbance of the rat by the

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[†] The thermometer manufactured by the Warren E. Collins Co., Boston, for use in the Benedict Roth Metabolism Machine has a brass jacket which can be screwed and cemented in place.

[2] Koehler, A. E., *J. Biol. Chem.*, 1932, **95**, 67.

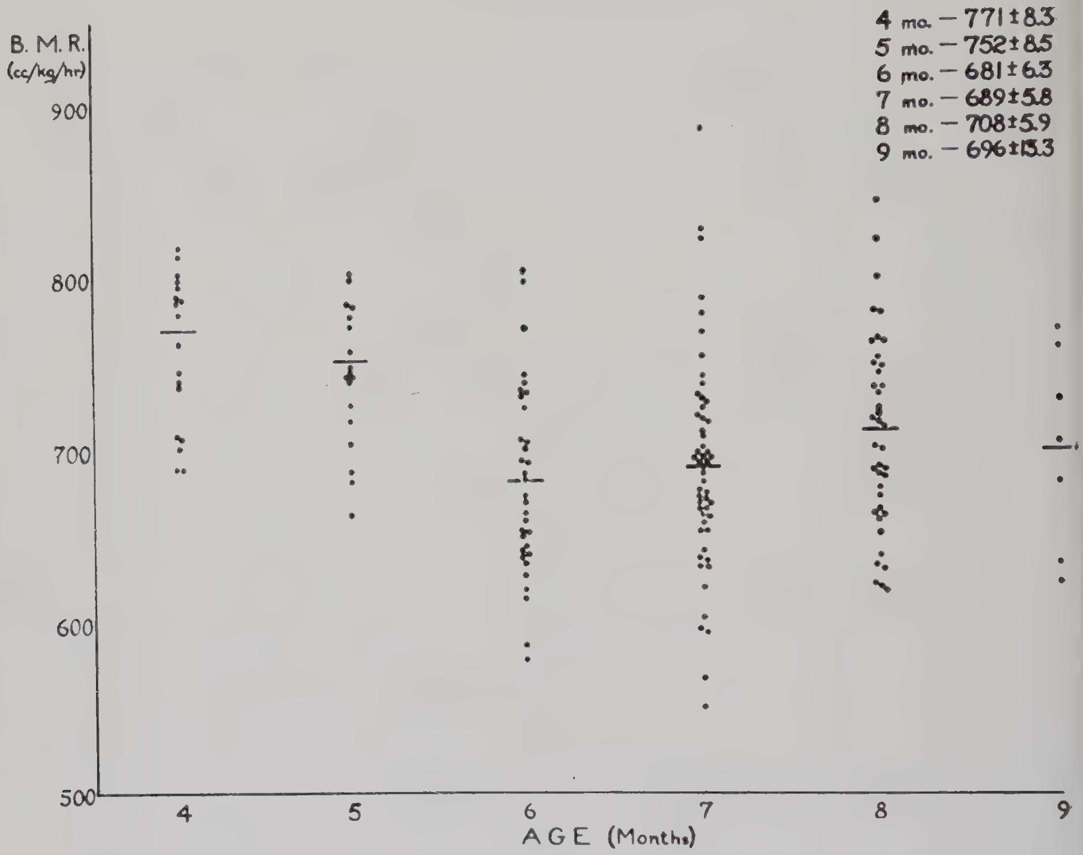

FIG. 2.

Relation of Oxygen Consumption to Age in Male Rats. Mean and probable error for each age level shown at upper right corner.

hiss of gas as it comes from a compressed oxygen tank.

At the tip of the tubular stainless steel writing lever is attached a free-swinging writing arm made of capillary glass tubing (see inset, Fig. 1). Such a writing point exerts a constant light pressure, determined solely by its own weight, against the kymograph drum. Fire-polishing the tip converts it into a smooth ball of low friction.

Use of the spirometer and writing point described above leads to a high degree of sensitivity, so much so that the slightest movement of the rat communicates itself as a gross hump to the otherwise smooth kymogram. Such precision affords an integral record of activity on the oxygen line; it also makes possible the measurement of oxygen consumption over so short an interval as 5 minutes. Such short periods were found to give lower and more consistent readings than the much more prolonged runs usually considered necessary.

Inscription of the tracing in this manner, however, introduces complications in measurement, since it brings about an interplay between 3 arcs, described by the drum's periphery, the spirometer bell, and the free-swinging writing arm. This requires that steps be taken to insure a constant, exactly reproducible spatial relationship between the kymograph and the basal metabolism machine, and that a measuring rule be made for the individual machine. The former control is easily made by fastening the machine and the kymograph to wooden bases so shaped that they fit together in only one position. The measuring rule is constructed in convenient form by transfer onto a plate of window glass of the record made by withdrawal of successive 5 cc volumes of air from the chamber into an accurate burette. The rulings are scratched on this glass plate with a diamond point and are then filled with colored wax; the transparent rule thus produced can be placed on each tracing and used to read off the oxygen consumption values accurately and quickly. Simultaneous ordinates for the signal magnet time record are transcribed onto the oxygen line of each tracing by manual operation of the drum and writing lever, after the test run.

In practice, then, the fasted rat is placed into the small wire mesh cage referred to above, and this is inserted into the animal chamber of the basal metabolism machine. Oxygen is admitted and allowed to flow out of the open stopcock and to bubble out of the spirometer until all air in the system is replaced by oxygen. Then the chamber is closed and at least 30 minutes are allowed for the animal to fall asleep and for the system to reach equilibrium. A 30-minute tracing is made and the best 5-minute period noted, along with an estimate of activity as determined from the smoothness of the tracing.

When the oxygen line indicates by an absence of any waves that the rat has been sleeping soundly throughout the recording period, all of the 5-minute readings give practically identical rates of oxygen consumption. Any movement which does occur elevates the succeeding readings in direct proportion to the intensity and duration of the activity; even a mere shift in position by a visibly sleeping animal increases the oxygen consumption for at least the next 5-minute period.

The graph of Fig. 2 shows the distribution of values, expressed as cc/kg/hr, according to age, in 172 determinations on 42 adult male rats. Only those observations made on sleeping animals are included, and all rats were housed in a constant temperature room at 26°C. Averages for the individual age groups show, with other observers, a progressive decline with age, reaching what appears to be a plateau at about 6 months. The mean oxygen consumption of the 133 determinations made in the 6-9-month age range is 692 cc/kg/hr ± 3.0 (P. E.), which may be taken as the basal metabolic rate obtained in "metabolic maturity." This figure is equivalent to 635 $Cal/m^2/24$ hr, using the formula, S. A. $= 9.1 \times W2/3$, for calculation of surface area.

Summary. 1. A method is described for determination of oxygen consumption in the rat. Its major advances are: (a) Humidity control, inducing sleep. (b) Sensitive recording method, which shows body movements on the oxygen line and makes feasible readings covering 5-minute periods.

2. Values obtained in 172 determinations on 42 male rats show a decline from 771 cc/kg/hr at age 4 months and 752 cc/kg/hr at 5 months to a plateau at 6 months and maintained to at least 9 months. The average for 133 determinations on rats of 6-9 months is 692 cc/kg/hr ± 3.0 (P. E.), or 635 $Cal/m^2/24$ hr.

15697

The Susceptibility to Furacin of Bacterial Strains Resistant to Sulfonamides or Antibiotics.*

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Furacin or 5-nitro-2-furaldehyde semicarbazone has been found to be both bactericidal and bacteriostatic for a large group of Gram-positive and Gram-negative bac-

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* This work has been aided by a grant from the Eaton Laboratories, Inc., Norwich, N.Y.

teria.[1] It has found practical application in the local treatment of infected areas when combined with carbowaxes and propylene glycol as a washable base.[2] In connection with its clinical application, it is of interest to determine whether or not organisms resistant to sulfonamides, penicillin or streptomycin are also resistant to furacin. The experiments reported in this paper have shown that pairs of strains respectively resistant and susceptible to sulfonamides, penicillin or streptomycin do not differ in their susceptibility to furacin.

Methods and Materials. Five pairs of coagulase-positive strains of *Staphylococcus aureus* were obtained—one member of each pair resistant and the other susceptible to sulfonamides. The resistant strains had been derived from the parent strain by continuous transfer in increasing sulfonamide concentrations. For streptomycin similar pairs were obtained comprising *Shigella paradysenteriae, Escherichia coli, Proteus vulgaris, Streptococcus viridans,* and *Staphylococcus albus.* The dysentery strains were obtained through the courtesy of Dr. Morton Klein of this laboratory. The resistant strain was obtained by culturing the organism in the presence of increasing concentrations of streptomycin. The others were obtained through the courtesy of Dr. C. W. Buggs of Wayne University School of Medicine. They were isolated from a patient who suffered from an abdominal gun shot wound and was treated with 4 g of streptomycin every 24 hours. The parent strains were isolated before therapy and the resistant ones at various times during therapy. Through the courtesy of Dr. John E. Blair, Hospital for Joint Diseases, New York, 3 pairs of coagulase-positive

TABLE I.
Growth of Organisms Susceptible and Resistant to Various Chemotherapeutic Agents in Dilutions of Furacin.*

Chemotherapeutic agent	Inhibitory concentration	Organisms	Dilutions of Furacin 10,000	25,000	50,000	75,000	100,000	250,000	500,000	750,000	1,000,000	Control
Sulfathiazole†	1/10,000	*S. aureus* 5A		—§			—	4	4	4	4	4
,,	1/1000	,, ,, 5E		—		—	—	4	4	4	4	4
Streptomycin	<16 γ/ml	*E. coli* 135A	—	—	4	4	4	4	4	4	4	4
,,	>5000 γ/ml	,, ,, 167A	—	—	4	4	4	4	4	4	4	4
,,	<32 γ/ml	*P. vulgaris* 196B	—	4	4	4	4	4	4	4	4	4
,,	>5000 γ/ml	,, ,, 198B	—	4	4	4	4	4	4	4	4	4
,,	200 γ/ml	*S. viridans* 135B	—	1	2	2	2	2	2	2	3	3
,,	>5000 γ/ml	,, ,, 179	—	1	2	2	2	2	2	2	3	3
,,	<1 γ/ml	*S. albus* 146B	—	—	—	—	—	3	3	3	3	3
,,	150 γ/ml	,, ,, 241B	—	—	—	—	—	3	3	3	3	3
,,	25 γ/ml	*S. paradysenteriae* (Flexner II)	—		—		—	4‖	4‖		4	4
,,	>1000 γ/ml	,, ,, ,, ,,	—		—		—	4‖	4‖		4	4
Penicillin‡	0.1 unit/ml	*S. aureus* 726	—	—	—	—	—	2	4	4	4	4
,,	>4 units/ml	,, ,, 726R		—	—	—	—	4	4	4	4	4

* 48-hour readings are given as representative of the results obtained.
† 5 pairs tested.
‡ 4 pairs tested.
§ No visible growth.
‖ Actual dilutions used were 200,000 and 400,000 instead of 20,000 and 500,000.

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[1] (a) Dodd, M. C., and Stillman, W. B., *J. Pharm. Exp. Therap.*, 1944, **82**, 11; (b) Dodd, M. C., *J. Pharm. Exp. Therap.*, 1946, **86**, 311; (c) Cramer, D. L., and Dodd, M. C., *J. Bact.*, 1946, **51**, 293.

[2] (a) Snyder, M. L., Kiehn, C. L., and Christopherson, J. W., *The Military Surgeon*, 1945, **97**, 380; (b) Dodd, M. C., Hartmann, F. W., and Ward, W. C., *Surgery, Gynecology, and Obstetrics*, 1946, **83**, 73; (c) Shipley, E. R., and Dodd, M. C., *Surgery, Gynecology, and Obstetrics*, in press; (d) Neter, E., and Lamberti, T. G., *Am. J. Surgery*, 1946, **72**, 246.

penicillin-resistant and susceptible strains of *S. aureus* were obtained. These strains were isolated before and after penicillin therapy.

The following procedure was used in testing the cross resistance of these strains:

All reagents were diluted either in casein hydrolysate or extract broth. The standard test inoculum for all strains was 200,000 bacteria per ml. The strains were checked for their resistance or susceptibility to penicillin, streptomycin or sulfonamides, respectively. All of the strains were tested against various dilutions of furacin to determine whether or not there was any difference in susceptibility to this drug of the resistant and susceptible strains. Daily visual observations of growth were made for 4 days and the results recorded as negative (no visible growth), doubtful, 1+, 2+, 3+ or 4+ growth. Casein hydrolysate medium was used for testing the penicillin and sulfonamide strains, extract broth for testing the streptomycin strains.

Results. In Table I representative results are given for each of the drugs tested. Not all of the results are given. Those omitted were essentially similar in character to those given in Table I. Except for some of the penicillin strains, a fairly high order of resistance to the various drugs tested was obtained. None of the paired organisms resistant to sulfathiazole, streptomycin or penicillin showed any change in resistance to furacin.

In most cases identical results with furacin were obtained with both the resistant and susceptible members of a pair of organisms. In some cases at the end of 48, 72, or 96 hours, differences were observed in the highest dilution inhibiting growth; that is, one member of a pair would show growth only at the next lower dilution. This reaction was just as likely to occur in the resistant as the susceptible strain of a pair of organisms.

Several experiments were tried with pairs of pneumococci[1b] resistant to sulfathiazole, acriflavine, atabrine and optochin. However, these strains grew at the highest possible concentration of furacin (1:5000), both under aerobic and anaerobic conditions, with minute inocula. The relative resistance to furacin, therefore, could not be ascertained.

Summary and Conclusions. Several Gram-negative and Gram-positive organisms resistant to sulfonamides, streptomycin and penicillin and the parent susceptible strains were tested in parallel for their susceptibility to furacin. No differences were observed between the resistant and susceptible strains in regard to their susceptibility to furacin. Resistance to penicillin, streptomycin or sulfathiazole does not result in any change to furacin resistance.

The authors wish to thank Dr. Morton Klein of this laboratory for suggestions and criticism during the course of the work, and Mr. Joseph Gots, also of this laboratory, for carrying out the experiments with pneumococci.

15698 P

The Corneal and Lenticular Changes Resulting from Amino Acid Deficiencies in the Rat.

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Corneal vascularization is a tissue change which may result from certain nutritional deficiencies.[1] Totter and Day[2] observed that this change resulted in the rat from deficiencies of tryptophane or lysine. Maun, Cahill and Davis found that corneal vascularization appeared in deficiencies of leucine[3] and of histidine[4] but no histological changes in the cornea were observed when rats were made deficient in phenylalanine.[5] Methionine[6] or protein deprivation[7] may similarly produce marked corneal changes, one of the most obvious of which is corneal vascularization. The suggestion has been made that corneal vascularization may be a reaction resulting from a deficiency of any of the indispensable amino acids or of protein.[8] In the following report is an account of the corneal changes which we have observed to occur in deficiencies of certain of the indispensable amino acids.

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[1] Dann, W. J., and Darby, W. J., *Physiol. Rev.* 1945, **25**, 326.

[2] Totter, J. R., and Day, P. L., *J. Nutrition,* 1942, **24**, 159.

Methods. Rats from a Wistar strain which were 25 or 26 days of age were placed in individual cages and given an experimental diet and water *ad libitum*. The control diets contained an amino acid mixture as used by Maun, Cahill and Davis[5] in their control diet. This supplied approximately 3 times the amount required for normal growth of each of the indispensable amino acids. The remainder of our control diet contained in each 100 g: salt mixture[9] 4 g, cod liver oil 2 g, cottonseed oil 3 g, choline chloride 0.2 g, riboflavin 1.6 mg, thiamin hydrochloride 0.4 mg, calcium pantothenate 2 mg, pyridoxin hydrochloride 0.4 mg and sufficient sucrose to make 100 g. Diets deficient in phenylalanine, leucine, isoleucine, arginine, histidine, threonine and valine were prepared by replacing the appropriate amino acid in the formula of the control diet with sucrose. Eight litters of 6 rats, one litter of 4, and one of 5 rats were distributed among the diets so that there were 7 rats from different litters on each diet except for 8 on the threonine-deficient diet. Biomicroscopic examination of the rats' eyes were made 3 or 4 times weekly. One rat from each group where corneal vascularization occurred was changed to the control diet to induce regression of the vascularization. Further details of technics used here were described in an earlier paper.[10] As before, we limit our use of the term corneal vascularization to the situation where there is an actual growth of capillaries in the cornea beyond the limits of normal variation.

Results. In all of the amino acid deficiencies investigated in this study some degree of corneal vascularization occurred. It was the most prompt and extreme in phenylalanine deficiency although the vascularization resulting from isoleucine and leucine deficiencies was nearly as extreme. Successively lesser degrees of corneal vascularization were observed in histidine, valine, threonine and least in arginine deficiencies. Of the 7 animals in each group on deficient diets the number which have showed corneal vascularization are respectively: phenylalanine 7, leucine 6, isoleucine 7, arginine 5, histidine 7, threonine 7 (of 8 rats), and valine 5. One of the control rats developed a few capillary loops into the edge of the cornea, beyond the usual limits of variation. Rats deficient in leucine, isoleucine, phenylalanine, histidine and threonine, when returned to the control diet to supply the lacking amino acid, underwent a prompt regression of vascularization until no corneal capillaries could be seen with the biomicroscope.

The observations described above as to the corneal vascularization occurring in leucine and histidine deficiencies are in accord with the findings of Maun, Cahill and Davis[1,2] in these deficiencies. Our findings in phenylalanine deficiency are in contradiction to those of Maun, Cahill and Davis,[3] since they reported that "neither the eyes of the deficient, nor those of the control animals showed alterations."

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[3] Maun, M. E., Cahill, W. M., and Davis, R. M., *Arch. Path.*, 1945, **40**, 173.

[4] Maun, M. E., Cahill, W. M., and Davis, R. M., *Arch. Path.*, 1946, **41**, 25.

[5] Maun, M. E., Cahill, W. M., and Davis, R. M., *Arch. Path.*, 1945, **39**, 294.

[6] Berg, J. L., Pund, E. R., Sydenstricker, V. P., Hall, W. K., Bowles, L. L., and Hock, C. W., *J. Nutrition*, in press.

[7] Hall, W. K., Sydenstricker, V. P., Hock, C. W., and Bowles, L. L., *J. Nutrition*, 1946, **32**, 509.

[8] Sydenstricker, V. P., Hall, W. K., Hock, C. W., and Pund, E. R., *Science*, 1946, **103**, 194.

[9] McKibben, J. M., Madden, R. J., Black, S., and Elvehjem, C. A., *Am. J. Physiol.*, 1939, **128**, 102.

[10] Bowles, L. L., Allen, L., Sydenstricker, V. P., Hock, C. W., and Hall, W. K., *J. Nutrition*, 1946, **32**, 19.

From the work reported or cited here it appears that corneal vascularization is a reaction which results in the rat from a deficiency of any of the indispensable amino acids or from protein deprivation.

To date, 4 of the 7 rats on the histidine-deficient diet have developed bilateral cataracts of varying size. It is to be noted that these 4 rats are all from different litters and that none of the rats on the other diets used in this study have shown any lenticular changes which could be seen with the biomicroscope.

Summary. In the rat, deficiencies of leucine, isoleucine, phenylalanine, histidine, threonine, valine, or of arginine, resulted in corneal vascularization. Four of 7 rats on a histidine-deficient diet developed lenticular opacities.

This study was aided by grants from the John and Mary R. Markle Foundation and the U. S. Public Health Service. Some of the amino acids used in this work were donated by Winthrop Chemical Company and by the Corn Products Refining Company.

15699

Chemical Changes in the Developing Turtle Embryo.

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It has been found in previous experiments[1] that the embryo of the snapping turtle (*Chelydra serpentina*) consumes fat in the amount of 61 mg per individual during the last 57 days of its development. Data obtained on the loss of dry substance during the same period, in conjunction with figures for fat loss and oxygen consumption, led to the assumption that relatively large amounts of protein were probably metabolized also. In the present study determinations of protein, carbohydrate, inorganic substances, dry substance and fat were made on turtle eggs at the beginning of development and on newly hatched turtles. The eggs used were collected from the oviducts of 3 large snapping turtles on June 11, 1946 and each egg was weighed individually. Fifteen of the eggs (5 from each clutch) were opened at once and the egg contents (albumen and yolk) of all 15 were put into a single vessel and dried at 110°C to serve for the determination of the initial chemical composition. The other eggs were incubated at room temperature and when the young turtles hatched (72-77 days) each was weighed and 15 specimens (5 from each clutch, as in the initial egg batch) were placed in a vessel and dried at 110°C. Eggs of as nearly as possible the same initial weights were used. The uniformity of the material is indicated by the fact that the average weight of the 15 eggs used for analysis of the chemical composition at the beginning of development was 11.13 ± 0.11 g (eggshells included) while the initial average weight of the 15 eggs which gave rise to the hatched turtles analyzed was 11.10 ± 0.09 g.

The 2 lots of dried material were each ground to a fine powder in a grinding mill and the following fractions were determined in aliquot parts by the methods specified: nitrogen according to Kjeldhal, using the customary factor of 6.25 to convert to protein; fat according to Kumagava-Suto;[2] total carbohydrate according to Dische and Popper[3] and inorganic substances by inciner-

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[1] Lynn, W. G., and von Brand, T., *Biol. Bull.*, 1945, **88**, 112.

[2] Kumagava, M., and Suto, K., *Bioch. Z.*, 1908, **8**. 212.

[3] D'sche, Z., and Popper, H., *Bioch. Z.*, 1926, **175**, 371.

TABLE I.
Chemical Changes in the Developing Embryo of the Snapping Turtle.

g	Egg	Hatched turtle	Difference
Avg fresh wt	9.89	8.01	—1.88
,, dry ,,	2.07	1.68	—0.39
,, protein content	1.15	0.93	—0.22
,, fat ,,	0.52	0.30	—0.22
,, carbohydrate content	0.14	0.16	+0.02
,, inorganic ,,	0.17	0.19	+0.02

ation.

Our results are summarized in Table I. It is obvious that the main substances consumed were fat and protein. Quantitatively, identical amounts of both groups of substances disappeared, but the greater amount of energy was obviously derived from the fat combustion. It will be noted that the sum of protein, fat, carbohydrate and inorganic substances amounts to about 95% of the dry weight in both batches. The 5% which is unaccounted for may be due to errors in the protein and carbohydrate determinations. It is questionable whether the customary conversion factor of 6.25 used in the protein determination is exactly applicable to turtle protein and it may well be that the carbohydrates present do not give exactly the same color intensity with the indole reagent as does the glucose solution used for comparison. The quantitative balance is sufficiently close, however, to prove that the developing snapper embryo derives its energy from the combustion of fat and protein. Similar observations have thus far been reported for only one other chelonian, the marine turtle *Thalassochelys corticata*.[4,5]

In our previous study[1] we found that a snapper egg consumes about 500 cc of oxygen during its development. The complete oxidation of 0.22 g of fat and 0.22 g of protein would require about 660 cc of oxygen. If some carbohydrate were synthesized from protein, the figure would be slightly lower, but would still be in excess of 500 cc. The discrepancy may be explained by the fact that the eggs used this year were somewhat heavier than those employed 2 years ago (11.1 g as compared with 9.9 g) and that their development required an average of 75 days as against 72 days.

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[4] Karashima, J., *J. Biochem. (Tokyo)*, 1929, **10**, 375.

[5] Nakamura, Y., *J. Biochem. (Tokyo)*, 1929, **10**, 357.

15700

Effect of Anemic Anoxia on Absorption of Isotonic Magnesium Sulfate from the Small Intestine.*

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It was shown by Northup and Van Liere[1] that even severe degrees of anoxic anoxia had no significant effect on absorption of magnesium sulfate from the small intestine of dogs. It had previously been shown,[2] however, that in anemic anoxia, produced by bleeding dogs 3.2% of their body weight, isotonic sodium chloride solution was absorbed from the small intestine more quickly than in control animals. The results were

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* Aided by a grant of the Ella Sachs Plotz Foundation.

[1] Northup, D. W., and Van Liere, E. J., *Arch. Internat. Pharmaco. et de Therap.*, 1939, **62**, 175.

[2] Van Liere, E. J., Northup, D. W., and Sleeth, C. K., *Am. J. Physiol.*, 1938, **124**, 102.

TABLE I.
Effect of Hemorrhage on the Absorption of Magnesium Sulfate.

Exp. No.	Magnesium absorption		Sulfate absorption	
	Control, %	Bled, %	Control, %	Bled, %
1.	—	21	—	11
2.	15	17	13	17
3.	24	17	24	18
4.	17	19	21	17
5.	5	26	10	28
6.	16	18	11	18
7.	10	17	9	17
8.	—	9	—	7
9.	18	—	11	—
10.	5	10	8	14
Avg	14	17	13	16*

* None of the differences between the means are significant by Fisher's *t* test.

statistically significant.

It was thought worthwhile to ascertain whether anemic anoxia (hemorrhage) would influence the rate of absorption of magnesium sulfate from the small intestine. It is known that this salt, if absorbed in sufficient quantities, may be quite toxic, so the problem is of practical as well as of academic interest.

Methods. Paired dogs as nearly alike in weight and age as possible were fasted 24 hours. One served as a control and the other was subjected to hemorrhage. Ten experiments were performed on a total of 20 animals.

The experimental animal was etherized, the femoral artery cannulated and blood withdrawn equal to 3% of the body weight. The artery was then ligated and the incision closed. Four hours were allowed for recovery from the ether and adjustment of the circulatory mechanism. During this interim, water was allowed *ad libitum.*

The animal was then given sodium barbital intravenously (300 mg/kg). The small intestine was exposed by a mid-line incision and practically the entire ileum used for a loop. The length of the loop was made the same as that in the control animal. It was washed out with isotonic glucose solution and filled with an isotonic solution of magnesium sulfate (3.25% anhydrous). After 90 minutes the fluid was removed from the loop and carefully measured. It was digested with concentrated HCl and the amount of sulfate determined gravimetrically by precipitation with barium chloride. The magnesium was determined by precipitation with 8-hydroxyquinoline.

The control animal was treated exactly as the experimental with the important exception, of course, that it was not bled.

Results. Table I shows the results obtained. There was no great difference of absorption of either magnesium or sulfate between the controls and those subjected to hemorrhage. The controls absorbed 13% of the sulfate and 14% of the magnesium, while the experimental animals absorbed 16% and 17% respectively. The differences in the means of the results were not statistically significant. There was a close parallelism between the absorption of magnesium and of sulfate. In all cases, save one, the same amount or more of fluid was recovered than was put into the intestinal loop. In one experimental animal 13 cc less was recovered than was put in. The greatest amount of secretion was 11 cc.

Discussion. The results clearly indicate that a pronounced hemorrhage does not make the intestinal epithelium more permeable to either the magnesium or the sulfate ion.

As pointed out previously[1] it is of distinct interest that an appreciable amount of magnesium sulfate is absorbed from the small intestine, even in a period of 90 minutes. This is not generally appreciated, although

Hirschfelder[3] reported that after 20-40 g of magnesium sulfate were administered to a human subject, no increase occurred in the magnesium content of the blood, but 40% was excreted in the urine in 24 hours. How much of the sulfate was absorbed was not stated.

Verzar and McDougal[4] pointed out that the rate of absorption of the sulfate ion is about the same as that of substances of equal weight and compares favorably with that of pentoses and certain hexoses. The data we are presenting show a parallelism between the rates of absorption of the magnesium and the sulfate ions. So far as we are aware, this has not been previously emphasized. Magnesium sulfate is widely employed by both practitioner and laymen and is probably one of the most commonly used household cathartics. A number of cases of magnesium poisoning[5,6] have rather recently been reported, some of which have proved fatal.

Summary. The rate of absorption of magnesium sulfate was studied in a series of barbitalized dogs each of which had suffered a hemorrhage equal to 3% of its body weight. The control animals absorbed 14% of the magnesium and 13% of the sulfate, whereas the experimental animals absorbed 17% and 16% respectively. The differences were not statistically significant. A fact not sufficiently appreciated is that the intestine is only relatively impermeable to this salt.

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[3] Hirschfelder, A. D., *J. Biol. Chem.*, 1934, **104**, 647.

[4] Verzar, F., and McDougal, E. J., *Absorption from the Intestine*, Longmans, Green & Co., London, 1936.

[5] Bryon, F. E., *J. Malaya Br., Brit. M. A.*, 1939, **3**, 100.

[6] Gens, J. P., *J. A. M. A.*, 1943, **123**, 1028.

15701

Estimation of Steroid Estrogens by Fluorimetry.*

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Estrogens are still generally determined by biological methods. As these present all the difficulties and uncertainties of the biological technic, considerable interest attaches to the development of an adequate chemical assay for the estrogens. During more than a decade repeated attempts have been made to elaborate a colorimetric procedure suitable to this purpose.[1–10] Most of this work has been based on the Kober reaction. The available colorimetric methods are, however, difficult in practice, of imperfect specificity, and often insufficient in sensitivity. A search in the field of fluorimetry for an adequate solution of this analytical problem would seem to be indicated. The proven high sensitivity of fluorimetric methods in vitamin assay encourages the hope that methods of this type could find usefulness in determination of hormones at biological level.

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* This paper was partly aided by a grant from the Max London Foundation administered by Dr. M. Bloom.

[1] Kober, S., *Biochem. Z.*, 1931, **239**, 209.

[2] Kober, S., *Biochem. J.*, 1938, **32**, 357.

[3] Cartland, F. G., Meyer, R. K., Miller, L. C., and Rutz, M. H., *J. Biol. Chem.*, 1935, **109**, 213.

[4] Venning, E. H., Evelyn, K. A., Harkness, E. V., and Browne, Y. S. L., *J. Biol. Chem.*, 1937, **120**, 225.

[5] Bachman, C., *J. Biol. Chem.*, 1939, **131**, 455.

[6] Bachman, C., and Pettit, D. S., *J. Biol. Chem.*, 1941, **138**, 689.

[7] Talbot, N. B., Wolfe, Y. K., MacLachlan, E. A., Karush, F., and Butler, A. M., *J. Biol. Chem.*, 1940, **134**, 319.

[8] David, K., *Acta brev. Neerland*, 1934, **4**, 64.

[9] Zimmermann, W., *Z. physiol. Chem.*, 1935, **233**, 257; 1936-37, **245**, 47.

[10] Pincus, G., Wheeler, G., Young, G., and Zahl, P. A., *J. Biol. Chem.*, 1936, **116**, 253.

The present paper describes a sensitive fluorimetric method for assay of the steroid estrogenic hormones on the basis of the fluorescence afforded when these substances are heated with phosphoric acid.

Method for estrone, estradiol and estriol in pure solutions. It has been known for some time that several natural estrogens—estrone, estradiol, estriol—produce on warming in concentrated sulfuric acid orange-colored solutions with green to blue fluorescence.[11–13] The fluorescence reaction has been employed widely as a qualitative test for estrogen in preparative work, and diagnostically as a qualitative test for pregnancy in the mare.[14,15] As sulfuric acid produces color under the same conditions, and in some cases fluorescence with a large range of substances, it seems not to have been considered worthwhile hitherto to attempt quantitative assay of estrogens on the basis of this reaction. In the method described below, this difficulty is avoided. By the use of phosphoric rather than sulfuric acid in the reaction solution, the interference of nonspecific response is largely eliminated.[16] Thus a quantitative test possessing a desired degree of specificity is afforded.

Reagents. 1. Phosphoric acid, s. g. 1.75, purest. 2. Crystalline estrogens.[†] 3. Ethanol, pure, redistilled from an all-glass still.

Special Apparatus. 1. Test tubes provided with ground-glass stoppers (capacity 15 cc, Pyrex). 2. Fluorimeter provided with a micro-cuvette (capacity 2 cc).

Procedure. A volume not exceeding 2 cc of the pure alcoholic solution of estrogen is evaporated from a test-tube over a water bath and oven-dried for one hour at 110°C. To the dry residue, 3.0 cc phosphoric acid is added from a burette. The test-tube, closed with ground-glass stopper, is transferred to a boiling water bath (98°) and heated in the dark for 30 minutes, the mixture being shaken during the first 2 min. of heating to insure the solution of the estrogen. Reaction is interrupted by cooling in tap water. The fluorescence is measured within an hour. The concentration of the estrogen solution is read against a concurrently prepared standard reference curve.

Prolonged exposure of estrogen in acid to light is avoided as light interferes with the development of the fluorescence and also causes gradual fading of developed fluorescence. In performing the reaction, care is taken to prevent accidental entry of water (spray or vapour) into the reaction mixture, as water sharply depresses the reaction and exerts a marked quenching effect. The reaction vessels should be sealed with close-fitting ground-glass stoppers, rubber and cork being unsatisfactory.

Experimental. Pure crystalline estrogens were used for the experiments. The fluorescence was measured in a 2 cc micro-cuvette with a Lumetron photofluorimeter[‡] using a Uviarc mercury vapor lamp equipped with primary filter as for riboflavin assay, and secondary orange filter which transmits freely above 5500 Å. The instrument was calibrated against fluorescein solution of known concentration.

Effect of heating time. The effect of heating time on the intensity of the fluorescent response of estrone and estradiol was determined for the time interval 5-30 minutes. The results in Table I demonstrate that the reaction proceeds regularly with time, rising rapidly in the first 10 minutes, and then less rapidly to a maximum at 20 minutes and is maintained at 30 minutes. Prolonged heating (60 minutes) led to loss of fluorescence. A heating time of 30 minutes seems therefore to be suitable and is employed in all following experiments.

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[11] Wieland, H., Straub, W., and Dorfmuller, T., *Z. physiol. Chem.*, 1929, **186**, 97.

[12] Marian, G., *Biochem. J.*, 1930, **24**, 1021.

[13] Schwenk, E., and Hildebrandt, F., *Biochem. Z.*, 1933, **259**, 1021.

[14] Cuboni, E., *Klin. Wchnschr.*, 1934, **8**, 302.

[15] Sala, S. L., cited in *Sex and Internal Secretion*, p. 892, Williams & Wilkins Co., 1939.

[16] Hestrin, S., and Mager, J., *Nature*, 1946, **158**, 95.

[†] Estrone and estradiol were kindly supplied by Dr. B. J. Brent, Roche Organon, Inc.; estriol was obtained through the courtesy of Dr. O. Kamm, Parke Davis Co.

[‡] Thanks are due to Prof. I. S. Friedenwald for his kind gift of a Lumetron apparatus.

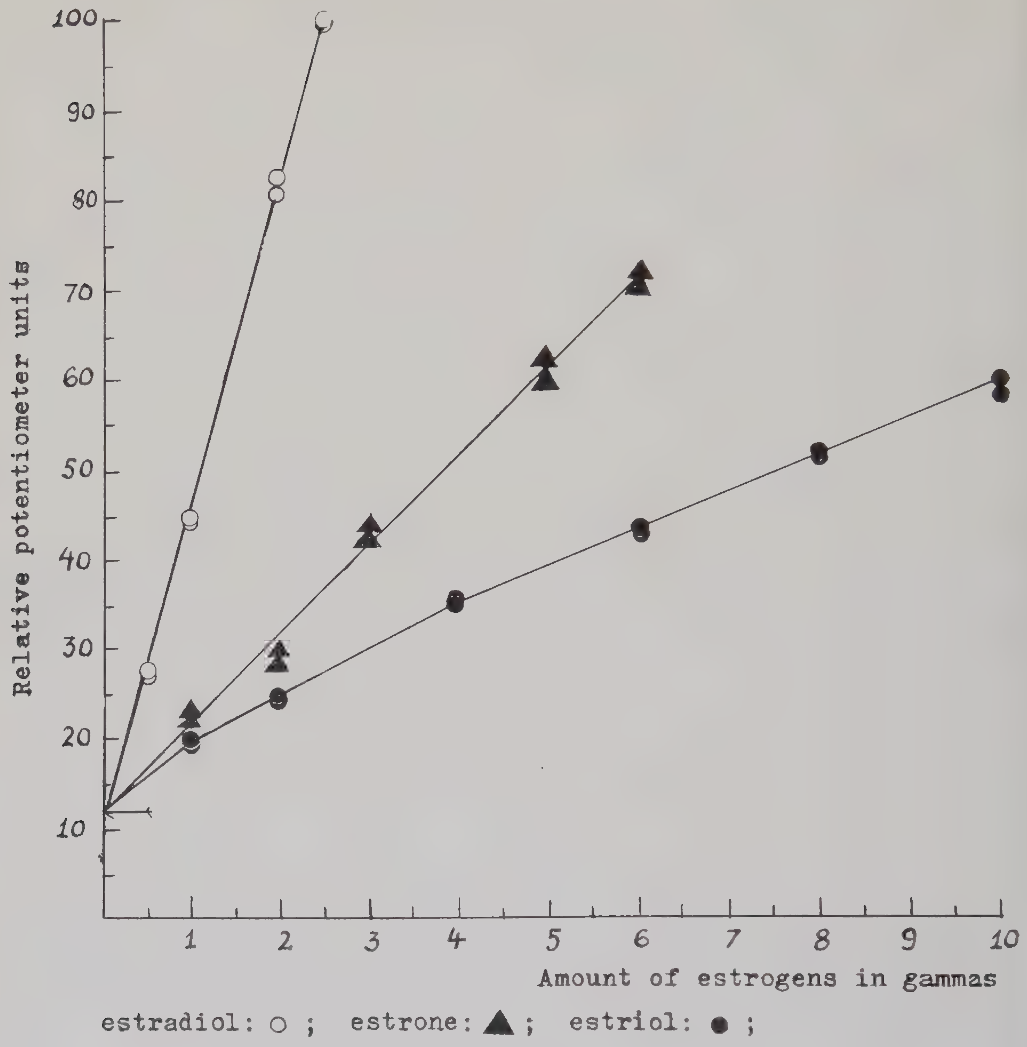

Chart 1.

Influence of estrogen concentration on fluorescence response.

← The ordinate intersect represents an intrinsic instrument factor.

TABLE I.
Effect of Heating Time in Phosphoric Acid on Fluorescence Reaction of Estrone and Estradiol.

	Fluorescence measurement in relative galvanometric units at different times			
Substances	5′	10′	20′	30′
Estrone, 3 γ	27.5	35.5	56.0	57.5
Estradiol, 1 γ	17.0	31.0	40.0	42.5

TABLE II.
Analyses of Estrogen Solutions by the Fluorimetric Method.

Estrogen	Conc. per cc in γ as given	Conc. per cc in γ as read	Error of estimation in γ
Estrone	0.00	0.0	0.0
	6.50	6.5	0.0
	8.50	8.3	—0.2
	9.00	8.9	—0.1
	9.30	8.95	—0.35
	10.00	9.75	—0.25
	10.00	9.80	—0.20
Estradiol	1.50	1.46	—0.04
	2.20	2.15	—0.05
	2.40	2.40	—0.00
	4.30	4.22	—0.08
Estriol	1.50	1.5	0.0
	1.50	1.65	+0.15
	4.00	3.8	—0.2
	4.00	4.0	0.0

Effect of estrogen concentration. Typical concentration response curves are shown in Chart 1. It can be seen that the intensity of fluorescence is a linear function of estrogen concentration to at least 2.5 γ of estradiol, and 5 γ of estrone. The curve for estriol seems to be slightly convex to about 2 γ, then straightens out and follows a linear course from 3 γ to at least 10 γ. The estriol solutions have a faint pink color; the estrone and estradiol solutions are virtually colorless. Weight for weight, estradiol is 3.6 times and estrone about 1.3 times as active in production of the fluorescence as estriol. The sensitivity of the method therefore varies with the estrogen determined.

Results obtained on solutions of pure estrogen in concentrations unknown to the authors at the time of the analyses are shown in Table II. In optimum ranges of estrogen concentration and when each test was performed in duplicate on 2 dilutions of the test solution, the mean error of estimation in a series of 30 determinations proved to be ±3%.

Preparation of standard curve. The instrument was calibrated against sodium fluoresceinate so that a solution of a concentration of 0.8 γ/cc rated near 40 on the galvanometer scale and precisely 100 on the potentiometric scale.

Calibrations were made both before each series of measurements and during measurements at intervals of 10 minutes. Standard curves were prepared from parallel measurements on graded estrogen concentrations so selected to fall within the potentiometric range 10-90. The ordinary scatter of results in a parallel replicate series can be seen in Fig. 1. Standard curves obtained on different days using the same batch of phosphoric acid fluctuated within ±5%. A daily check of the standard curve is recommended for accurate work.

Specificity. Effective use of the fluorescence reaction as the basis of estrogen assay in biological materials is dependent on proof of the reaction's specificity as between different ether-soluble biological substances which tend to accompany the estrogens in usual procedures for their extraction.

Nonestrogenic mononuclear urinary phenols (tyrosine, phenol, and cresol) showed in an amount of 1 mg neither fluorescence nor color reaction under the conditions of the test.

The behaviour of 13 sterols§ listed in order of decreasing activity is recorded in Table III. It may be seen that of the examined nonestrogenic sterols only desoxycorticosterone and testosterone have sufficient activity to produce a false positive estrogen reading under the conditions of the test. As

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§ The authors are indebted to Dr. F. Bergman, Daniel Sief Research Institute, Rehovot, for a kind gift of sterols.

TABLE III.

Correlation of Chemical Structure and Fluorescence Response to Heating in Phosphoric Acid.*

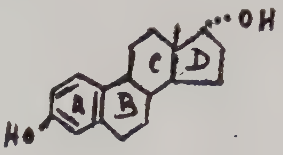
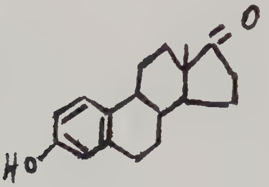
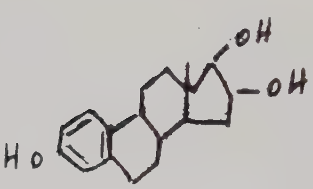
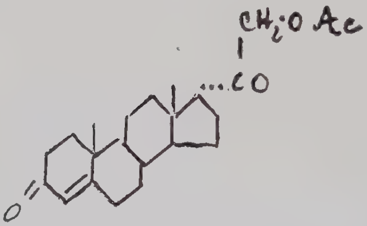
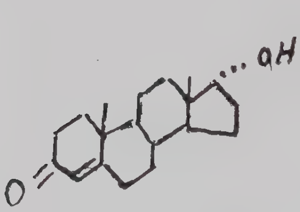
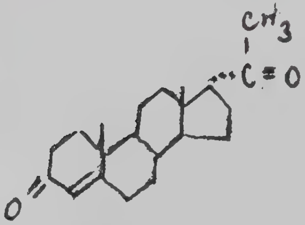
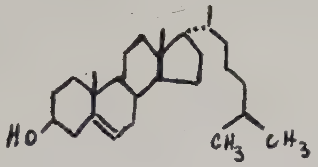
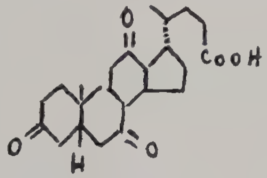
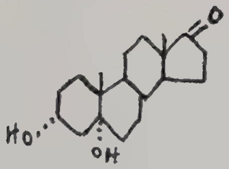
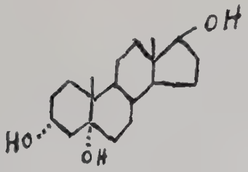
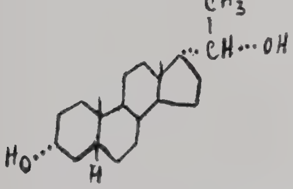
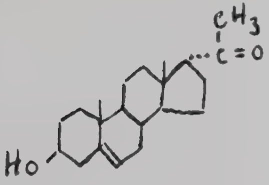
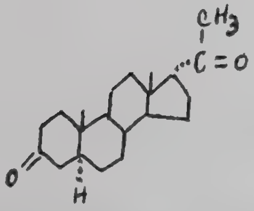
No.	Substance	Structural formula	Fluorescence activity per γ relative to estrone = 1.
1	Estradiol		2.7
2	Estrone		1.0
3	Estriol		0.75
4	Desoxycorticosterone-acetate		0.3
5	Testosterone		0.1
6	Progesterone		0.01

TABLE III. (Continued).

No.	Substance	Structural formula	Fluorescence activity per γ relative to estrone = 1
7	Cholesterol		0
8	Dehydrocholic acid		0
9	Androsterone		0
10	Androstanediol		0
11	Pregnanediol		0
12	Pregnenolone		0
13	Allo-pregnanedione		0

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* The structural formulæ conform to the conventions adopted by H. Selye (*Encyclopedia of Endocrinology*, Franks Publishing Co., Montreal, 1943).

neither of these 2 possible interferants is a phenol, their separation from the urinary estrogens presents no great difficulty.

In test-tube experiments on a random collection of substances, the following results were recorded. Tryptophane and quinine showed marked fluorescence. Stilbestrol, catechol, all amino acids other than tryptophane, glucose and polyglucosides, several purines and polyhydric alcohols showed no reaction. Carbohydrates containing a nonglucose sugar constituent gave amber reactions but no fluorescence.[17]

Discussion. The fluorescence reaction described seems suitable for use as the basis of an estrogen assay. Furthermore this reaction may afford also a basis of assay for certain nonestrogenic sterols, *e.g.* testosterone and desoxycorticosterone acetate. The fluorescence response of these latter, though smaller than that of the estrogens, is readily measurable at a level of about 10 γ. On the other hand, the minimum amounts of estrogen that can be determined in terms of fluorescence reaction within an error range of $\pm 3\%$ are for estrone 1 γ or 10 I.U., for estradiol 0.5 γ or about 5 I.U., and for estriol 1 γ or about 0.01 I.U. Thus the sensitivity of the fluorescence assay in the case of estriol exceeds that of the biological test and in all the cases exceeds that of known colorimetric procedures.[1–10]

When estrogens are heated with sulfuric acid, a fluorescence exceeding in intensity that afforded by phosphoric acid is obtained. In principle, therefore, sulfuric acid might seem preferable to phosphoric acid as the reaction agent. When sulfuric acid is used, however, blank fluorescence of the reagent complicates the reading, color changes obscure the determination, the results are less reproducible, and greater technical inconvenience is encountered. For quantitative work, phosphoric acid seems decidedly to be preferable.

The experiments reported show that fluorescent response to heating in phosphoric acid is a specific characteristic of certain sterols. A consideration of the structural formulae of the investigated sterols (Table III) leads to certain generalizations concerning structural features which mediate the production of fluorescence under the conditions of this test. It is evident from the data that the investigated sterols fall into 2 groups:

(a) Substances Nos. 1-6 which show a definite fluorescent response.

(b) Substances Nos. 7-13 which show no measurable fluorescent response.

All the substances of Group (a) possess a conjugated double-bond system and have this system in ring A of their structure. Two types of conjugated double-bond systems are exemplified in this group: aromatic or polyenoid (C:C·C:C), *e.g.* substances Nos. 1-3; and catioenoid (C:C·C:O), *e.g.* substances Nos. 4-6. On the other hand, no member of Group (b) possesses a conjugated double bond, although some of the members of this group do possess one or more nonconjugated double bonds. It is suggested therefore that the presence in the sterol molecule of a conjugated double bond is a prerequisite of the fluorescent activity. From the fact that the magnitude of the response of different substances in Group (a) varies markedly, it must be concluded that the magnitude of a fluorescent response is determined not merely by the presence of a conjugated double bond but also by other characteristics of the molecule. It is evident that the presence and position of polar groups exerts an important influence on the magnitude of the fluorescent response. High activity such as the urinary estrogens exhibit appears to be associated with aromatic phenol character in ring A and presence and position of oxygen bonds in ring D (Table III).

As may be seen below, the biological activity and fluorescent response of the investigated estrogen trio are correlated, though by no means perfectly.

Relative activity	Estriol	Estrone	Estradiol
Estrogenic	0.01	1	5
Fluorimetric	0.75	1	2.7

Since the ratio of estrogenic activity to fluorimetric response per gamma is a variable, it is clear that the application of the reaction to estrogen assay in a material which contains several estrogens must entail prior separation of the different components. Several recommended fractionation technics fortunately meet this requirement.[6,7,18-20] It may be noted here that the nonsteroid estrogen, stilbestrol, does not produce fluorescence under the conditions of the test.

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[17] Hestrin, S., and Mager, J., 1946, unpublished.

The fluorimetric method is simple, the reproducibility of the result is good, and the specificity of the test promises to be as good as or better than has been obtained with the previously described colorimetric methods. Further study of the analytical possibilities of this assay seems therefore to be desirable. A survey of the effect of phosphoric acid on a larger number of sterols and related substances with a view to elucitating the relation of sterol structure to fluorescent response has accordingly been undertaken. At the same time the application of the fluorimetric method to estrogen determination in blood and urine is being examined. The results will be reported elsewhere.

Summary. 1. A method for estimation of estrone, estradiol, and estriol in pure solution is based on the observation that these substances afford products with a green fluorescence when heated in phosphoric acid.

2. The fluorescent response of the urinary estrogens is over a wide range a linear function of their concentration. The error of the fluorimetric assay in suitable ranges of estrogen concentrations (estradiol, 0.5-2.5 γ; estrone, 1.0-5.0 γ; and estriol, 1.0-10.0 γ) does not exceed $\pm 3\%$.

3. Sterols which do not contain a conjugated double bond, some naturally occurring nonsteroid aromatic substances, and many common constituents of biological material give no fluorescent response under the conditions of the test.

4. Estrogen fluorimetry appears to compare favorably as to sensitivity, specificity and simplicity with previously described methods of estrogen assay.

The authors wish to express their indebtedness to Prof. B. Zondek, for his kind interest and support of this investigation.

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[18] Mather, A., *J. Biol. Chem.*, 1942, **144**, 617.

[19] Pincus, G., and Pearlman, W. H., *Endocrinology*, 1942, **31**, 507.

[20] Stimmel, B. F., *J. Biol. Chem.*, 1946, **162**, 99.

15702 P

Induction of Renal Glomerular Lesions by Urethane in Inbred Mice Susceptible to Spontaneous Glomerulonephritis.*

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Mice of the NH strain develop spontaneous glomerulonephritis. Histologically the glomeruli are similar to those of the human chronic disease.[1]

Dunn and Larsen[2] reported that urethane induced hyaline alterations in the glomeruli of mice of the Strong A strain, whereas similar changes were lacking in the glomeruli of other stocks of mice following the administration of this drug.

The work of Dunn and Larsen suggested to the authors that urethane might provoke interesting changes in the glomeruli of mice which are susceptible to the development of spontaneous glomerulonephritis. Twenty-one NH mice received 1 mg per g of body weight of urethane (anesthetic dose) in

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* This investigation has been aided by a grant from the Life Insurance Medical Research Fund. The authors are indebted to Miss Marthella Frantz, Miss Elnora Stahn, and Mrs. Sonia Braun for valuable technical assistance.

[1] Kirschbaum, A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 280.

[2] Dunn, T. B., and Larsen, C. D., *Fed. Proc.*, Part I, 1946.

aqueous solution once each week by subcutaneous injection. The mice were 6 to 8 weeks of age when the experiment was begun.

Between 5 and 6 months after the series of injections was started 11 of the mice became grossly edematous. The plasma protein levels of these animals were depressed (values of less than 5.2 g%,[3] the lowest level obtained for the mice without edema), the urine contained albumin, and blood urea nitrogen was elevated (range of from 50 to 265 mg% as compared with a normal of 18-27 mg%).[4] In one case there was a gross hyperlipemia; the plasma cholesterol value was 430 mg% as compared with a normal for mice of approximately 100 mg%.[5] All the injected mice were anemic. The degree of nitrogen retention, but not of anemia, could be correlated with the extent of the pathologic alterations observed in the glomeruli.

The essential pathologic changes in the glomeruli were thickening of the capillary basement membrane (azo-carmine stain), capillary thrombosis, and closure of many capillary tufts resulting from the basement membrane alteration. The affected glomeruli were smaller than those observed in spontaneous glomerulonephritis of NH mice, more glomeruli were normal, and except in an occasional glomerulus there was nothing to suggest endothelial proliferation. Histologically the glomeruli resembled those of human lipoid nephrosis.[6] In one case most of the glomeruli were extensively infiltrated with polymorphonuclear leukocytes.

Of the 21 mice injected with urethane, either edema or histologic evidence of renal disease and nitrogen retention were observed in 17. One animal was not nephritic as judged by gross, histologic and biochemical evidence; 3 were without renal disease grossly and the kidneys were not studied histologically because of postmortem degeneration.

Spontaneous glomerulonephritis appears rarely before the age of 8 months in NH mice. Of 53 cases in a total of 199 controls only 5 appeared before 8 months of age. There is no doubt that the glomerular lesions were induced by urethane; the effect of urethane on the kidney was confined to the glomerulus.

Urethane is a capillary poison.[7-9] In NH mice the glomerular capillaries especially seem to be susceptible to its effects. The drug also induces lung tumor development in mice,[10] and most of the animals of this experiment developed multiple lung adenomas. It is likely that the anemia was not entirely the result of the renal disease. Urethane depresses the myeloid tissues of mice,[11] and, furthermore, the injected animals failed to gain in weight to the same extent as normal mice. These animals might belong in the same category as underfed mice, which are characteristically anemic.

Summary. Urethane induced glomerular lesions in NH mice, an inbred strain which is susceptible to the spontaneous development of glomerulonephritis. Mice with the urethane-induced glomerular alterations exhibited renal insufficiency, albuminuria, edema, and depressed plasma protein levels. One case evidenced hypercholesterolemia.

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[3] Koch, F. C., and McMeekin, T. L., *J. Am. Chem. Soc.*, 1924, **46**, 2066 (method for determining plasma proteins).

[4] Karr, W. G., *J. Lab. Clin. Med.*, 1924, **9**, 329 (method for determining blood urea nitrogen).

[5] Bloor, W. R., *J. Biol. Chem.*, 1928, **77**, 53 (method for determining plasma cholesterol).

[6] Bell, E. T., *Am. J. Path.*, 1938, **14**, 691.

[7] Krogh, A., and Harrop, G. A., *J. Physiol.*, 1920, **54**, CXXV.

[8] Landis, E. M., *Am. J. Physiol.*, 1927, **81**, 124

[9] Doljanski, L., and Rosin, A., *Am. J. Path.*, 1944, **20**, 945.

[10] Nettleship, A., and Henshaw, P. S., *J. Nat Cancer Inst.*, 1943, **4**, 309.

[11] Engstrom, R., Kirschbaum, A., and Mixer H. W., *Science*, in press.

15703

Coproporphyrinuria (Type III) in Acute Poliomyelitis.*

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The recent epidemic of poliomyelitis in Minneapolis has provided opportunity to investigate the urinary coproporphyrin excretion in this disease. The need for such a study was suggested by a number of facts relating to the normal and pathological physiology of the porphyrins. Chief among these was the observation of Klüver,[1,2] that the white matter of the central nervous system of warm-blooded animals regularly contains a small amount of coproporphyrin. Klüver's study[†] indicated that this was probably the Type III isomer, at least from the standpoint of its solubility characteristics. Our own studies bearing on this point, to be described in detail elsewhere, have likewise indicated the presence of the Type III isomer, on the basis of the differential precipitation or "fluorescence quenching" method.[3]

In addition to the physiological occurrence of coproporphyrin in the central nervous system, the markedly increased urinary excretion of the Type III isomer in lead and arsenic poisoning, and, in association with uroporphyrin, in intermittent acute porphyria (diseases in which the nervous system is commonly affected), constituted additional reasons for investigating the porphyrin metabolism in poliomyelitis. This disease has in fact been confused clinically with acute porphyria,[4] an additional instance of the latter type having come to our attention in recent weeks.

Still another reason for investigating the urinary coproporphyrin in poliomyelitis is the occurrence of unexplained hypertension in this disease, especially in the bulbar type. This has been observed rather frequently in the present epidemic. So far as can be determined, hypertension in poliomyelitis has been mentioned specifically in but one report in literature (Salus[5]) although it has been referred to in association with lesions of the brain stem.[6] It is well known, of course, that hypertension is a relatively common manifestation of intermittent acute porphyria, with or without bulbar manifestations, although the relationship is as yet unexplained. Carrié[7] classifies the hypertension of porphyria, and of lead poisoning, as vasospastic in type, designating it as "porphyrinopathic."

The present material consisted of 64 cases of poliomyelitis studied at various stages of the disease, and representing various types and degrees of severity. One hundred and ninety determinations of total coproporphyrin were made in these cases, on aliquots of 24-hour urine samples, a modification of Fikentscher's method[8] being used.[‡] Fifty-one isomer analyses[‡] were made by means of the differential precipitation of the esters of coproporphyrin I and III in 30-35% acetone in the cold.[3,9] The results obtained

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* Aided by a grant from the John and Mary R. Markle Foundation, New York City, and the National Foundation for Infantile Paralysis, Inc.

[1] Klüver, H., *J. Psychol.*, 1944, **17**, 209.

[2] Klüver, H., *Science*, 1944, **99**, 482.

[†] Personal communication.

[3] Schwartz, S., Hawkinson, V., and Watson, C. J., *Science*, 1946, **103**, 338.

[4] Waldenström, J., *Acta Psychiatrica et Neurologica*, 1939, **14**, 375.

[5] Salus, F., *Klin. Wchnschr.*, 1932, **11**, 1542.

[6] Page, I. H., and Corcoran, A. C., *Arterial Hypertension. Its Diagnosis and Treatment*, Year Book Publishers, Chicago, 1945, p. 20.

[7] Carrié, C., *Die Porphyrine, Ihr Nachweis, Ihre Physiologie und Klinik*, Leipzig, G. Thieme, 1936.

[8] Fikentscher, R., *Biochem. Z.*, 1932, **249**, 257.

[‡] Details of these procedures will be given in a forthcoming publication.[9] Of the two alternative methods of isomer analysis, A and B, the latter was used in the present study.

[9] Schwartz, S., Hawkinson, V., Cohen, S., and Watson, C. J., *J. Biol. Chem.*, in press.

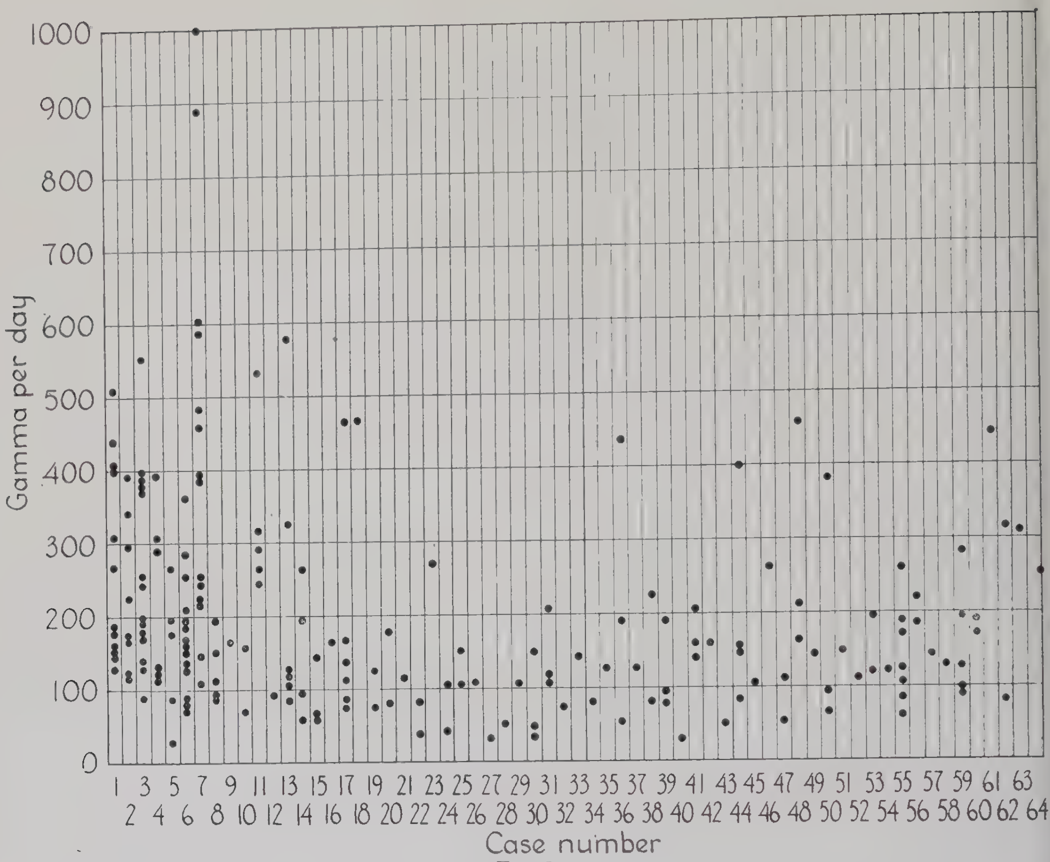

FIG. 1.

Values for total coproporphyrin in γ per 24 hours, as determined 190 times in 64 cases.

have been sufficiently uniform that there can be little doubt about the essential phenomenon which they represent, whatever its significance. The purpose of the present report is to record these observations.

In Fig. 1, the results of the total coproporphyrin determinations are shown as carried out on the 24-hour urine samples. In Fig. 2, 51 isomer analyses as carried out on 38 cases, have been plotted in terms of both total coproporphyrin and percentage of the Type III isomer, as compared with the normal range (the shaded area in the left lower corner of Fig. 2).

Data have been presented,[10] and these will be considered in more detail elsewhere, which reveal that the normal range of urinary coproporphyrin in adult human beings is from 20-100 γ per day, of which 8 to 35% is the Type III isomer, the remainder being Type I. Comparison of these figures with those given above in Fig. 1 and 2 reveal the frequency of increase of the total coproporphyrin and of the proportion of the Type III isomer which characterize the urine in cases of poliomyelitis.

Since the possibility existed that some peculiarity of the urine in these cases might be affecting the differential precipitation technic in such a way that Type I isomer was behaving in part like Type III, it was clearly necessary to control the observations by means of isolation of the crystalline coproporphyrin ester and determination of its melting point. This has been done from the pooled final solutions used for isomer analysis, as obtained from 19 urine samples;

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[10] Watson, C. J., Proc. Am. Soc. Clin. Invest., 1946, *J. Clin. Invest.*, in press.

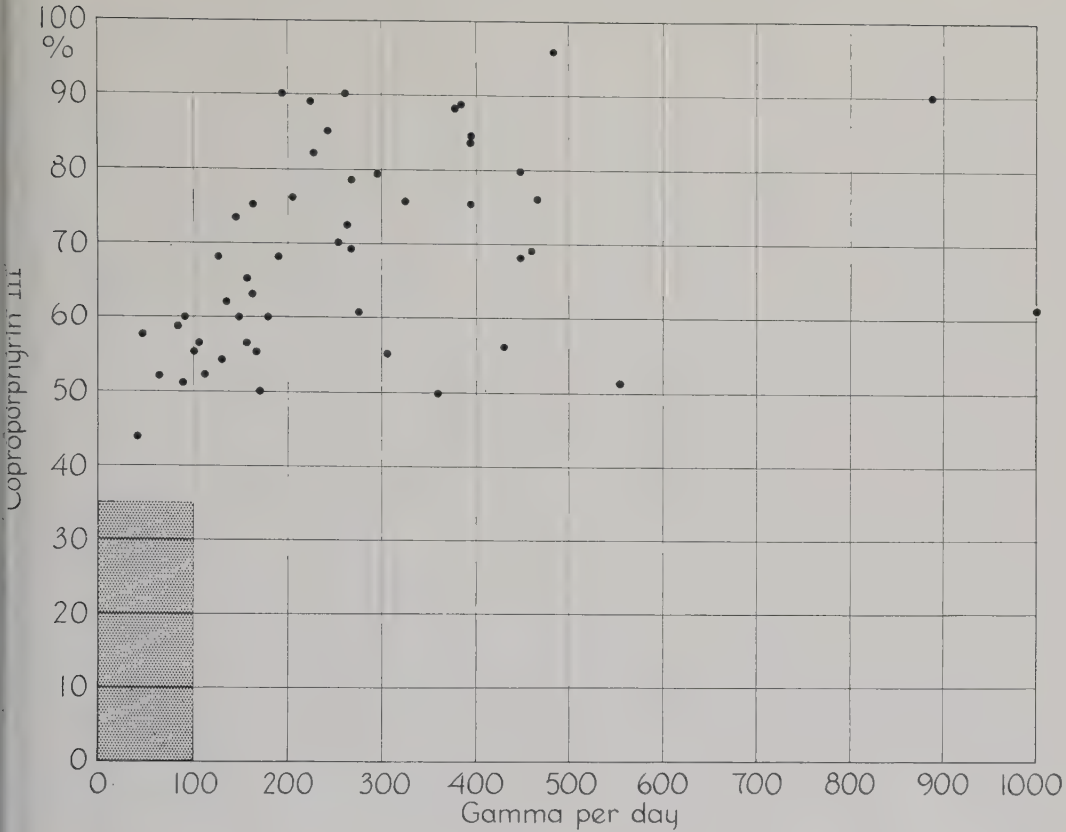

FIG. 2.

Total coproporphyrin (abscissa) and percentage of type III isomer (ordinate) in 38 cases (51 analyses). The shaded area in the lower left corner represents the normal range.

also by direct isolation[11] from five 24-hour urine samples, 2 from Case 7, and 1 each from Cases 1, 2 and 59 (case numbers refer to Fig. 1).

The results of these isolations are given n Table I, in which it is evident that the Type III coproporphyrin isomer is clearly present in excess.

One case, which was not included among the above, deserves special comment. In this instance (L.H., ♂, 32), despite a value of 642 γ for total urinary coproporphyrin, the isomer analysis revealed but 18% of Type III. This patient differed from all the others, however, in having a mild jaundice, and in having been exposed to 2 cases of jaundice among the members of her own family, about one month previously. In addition to jaundice, she exhibited a tender liver for a short period; also, evidence of hepatic functional impairment including urobilinogenuria, positive cephalin-cholesterol flocculation and thymol turbidity tests, and abnormal bromsulfalein retention. While all the latter might possibly be regarded as due to toxic injury of the liver secondary to poliomyelitis, the presence of jaundice, the history of close contact with infectious jaundice, and the tender liver, strongly favored a specific virus hepatitis. Yet there was also evidence suggestive of poliomyelitis. The neck flexion sign was strongly positive, there was muscle weakness, especially in the deltoids and triceps, and the spinal fluid contained 17 cells per cu mm (100% lymphocytes). Although, as noted above, the majority of the increased coproporphyrin was Type I, the actual amount of coproporphyrin III in the 24-hour sample, *i.e.*, 117 γ, is far above the normal. Thus, it is evident that both isomers

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[11] Grinstein, M., Schwartz, S., and Watson, C. J., *J. Biol. Chem.*, 1945, **157**, 323.

TABLE I.
Percentage of Type III Isomer, and Melting Points of Crystalline Methyl Esters Isolated from Various Cases in the Series.

Case No.	Total coproporphyrin in γ per 24 hr	% of Type III isomer	Crystal habitus	Melting point (°C) of crystalline methyl ester: 1st crop	1st recryst.	2nd recryst.	Melting point (°C) of Cu complex of methyl ester: 1st crop	Recryst.
1. (H.M.)	441	80	Rosettes of prisms*	130-151	128-135	‡	200-210	201-209
2. (I.D.)	390	75	” ” ”	130-142	130-132	‡		
59. (W.S.)	378	88	” ” ”	131-143	130-131	‡		
7. (K.P.)	1000	62	” ” ”	160-183	152-160	130-132	199-209	200-206
7. (K.P.)	890	90	” ” ”	130-141	130-134			
45-63†			” ” ”	150-183	140-142	130-132		

* The tetramethyl ester of coproporphyrin III crystallizes characteristically in rosettes of straight prisms which exhibit dimorphism in melting point: 135°; 144°C; 167-170°C.[14]

† Pooled final solutions used for total coproporphyrin determinations.

‡ Pooled crystals from cases 1, 2, and 59, were converted to the Cu complex[14] which crystallized from $CHCl_3$: CH_3OH after preliminary chromatographic purification.[11] The Cu complex of coproporphyrin III methyl ester melts at 206-207°C (corrected). All of the above melting points are corrected.

were increased, a finding which is fully compatible with the belief that the patient had both diseases simultaneously.

Discussion. The data thus far obtained do not indicate the existence, in poliomyelitis, of a correlation of the degree of increase of the urinary coproporphyrin with severity of the disease, prognosis as to death or recovery, or extent of residual paralysis; or as to type of disease, whether bulbar, spinal respiratory, or mixed. Nevertheless, in the 10 fatal cases which have been studied, all the determinations of the total coproporphyrin and the percentage of Type III isomer were significantly, and as a rule markedly, elevated regardless of the stage of the disease. In a number of instances, high values have been encountered for many days and in some cases for a number of weeks beyond the febrile period, likewise in some cases who have had a relatively mild attack and are now recovering. The majority of the values below 100 γ per 24 hours, as noted in Fig. 1, were obtained during the convalescent period. With reference to Fig. 1, it may also be noted that the levels of coproporphyrin excretion in an individual patient have often shown wide variation.

For example, in Case 1, the first determination made on the 4th day of the disease was 186 γ after which it fell to 125 γ and 154 γ on the 6th and 8th days after onset, then rose to 306 γ on the 9th day only to fall again to approximately 150 γ for 3 days, with a further rise to 400 γ from the 13th to 16th days after onset. On the 19th day the value was 264 γ and this was followed by a rise to the highest level of 505 γ on the 21st day. The last value obtained was 441 γ on the 24th day, death ensuing on the 27th day.

By contrast, in Case 2, the highest levels were obtained on the 3rd through the 9th days of the disease with a progressive fall through the 16th day of the disease to a level of 120 γ, death ensuing on the 19th day after onset.

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[14] Fischer, H., and Orth, H., *Die Chemie des Pyrrols*, Bd. II, 1 Hälfte, Akad. Verlagsgesellsch. Leipzig, 1937.

In Case 7, in which the highest levels were noted, the first determination, on the 9th day of the disease, was 216 γ, after which it increased to 392 γ on the 11th, 455 γ on the 13th, 890 γ on the 14th, then fell to an almost normal level of 105 on the 20th day only to rise again to a 1000 γ level on the 23rd day, without any marked change in the clinical condition of the patient. The level then again fell to 146 γ on the 26th day of the disease with exitus on the 30th day after onset.

In Case 3, determinations were made from the 20th to the 44th day of the disease, with levels ranging from 195 γ on the 20th day to a high of 550 γ on the 26th day, with several subsequent rises and falls and finally a decrease to the normal range on the 44th day after onset. This patient went on to a good clinical recovery.

It is thus apparent that the urinary coproporphyrin values have not been of assistance in prognosis nor could we correlate them with the current clinical status of the patient. Insofar as hypertension was concerned, there was also little, if any, correlation. It was true that in nearly all cases with elevated blood pressure in which porphyrin determinations were made, increased amounts were found. The only 2 exceptions were in pregnant women whose acute attacks were 3-4 months in the background and whose hypertension was believed to be on the basis of toxemia of pregnancy. There were many individuals, however, whose blood pressure was normal in spite of markedly increased porphyrin values. If the elevated blood pressure is "porphyrinopathic" it would have to be assumed that constitutional or other factors were responsible for the differences encountered. In one instance the blood pressure and porphyrin values were noted to decrease concomitantly, but in other cases marked fluctuations in blood pressure were recorded without evident correlation with the urinary porphyrin values.

Fever[12,13] and epidemic hepatitis,[10–13] as well as ordinary hepatic functional disturbances, whether parenchymal or mechanical in type,[10–13] have been shown to be associated with an increase of the Type I coproporphyrin isomer in the urine. Until now, infectious disease has not been found to cause an increased excretion of coproporphyrin III. It is of much interest in this connection that the increase in cases of infectious hepatitis is regularly due to the Type I isomer,[10] yet the virus of this disease is similar in some respects to the virus of poliomyelitis, both being relatively resistant and both being found in the gastrointestinal tract. Thus, it would appear logical to seek for an explanation of the difference in porphyrin excretion in the 2 diseases in the effect on the organism of the neurotropic as versus the hepatotropic nature of the respective viruses. The possibility must be considered that the excessive coproporphyrin III excretion observed in the present cases of poliomyelitis is in some way related to a disturbance of the coproporphyrin of the central nervous system. Another possibility is that it is derived from myohemoglobin, as a result of the disturbance in muscle physiology. This appears unlikely in view of the fact that marked increases have been noted in cases of relatively pure bulbar type with little or no muscle paralysis, also in early cases prior to any demonstrable muscle weakness.

Inspection of the isomer data shown in Fig. 2 reveals that while coproporphyrin III is clearly preponderant there is also, in many instances, a distinct increase of the Type I isomer. The simultaneous occurrence of marked urobilinogenuria in a number of these cases suggests that the increase in coproporphyrin I may be due to hepatic functional impairment.

Further studies are in progress with respect to the significance of these observations. The increased excretion of coproporphyrin III establishes a biochemical difference between 2 specific virus diseases, *i.e.*, poliomyelitis and infectious hepatitis. It is desirable to ascertain whether other neurotropic virus affections are similarly characterized, and whether virus diseases of dissimilar type, such as atypical pneumonia or psittacosis, if they exhibit any increases in

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[12] Watson, C. J., *J. Clin. Invest.*, 1936, **15**, 327.

[13] Dobriner, K., *J. Biol. Chem.*, 1936, **113**, 1.

urinary porphyrin at all, are, like infectious hepatitis, characterized by an increase of the Type I isomer.

Summary and Conclusion. A considerable excess in the excretion of coproporphyrin III has been commonly noted in a series of urine samples from cases of acute poliomyelitis. The total urinary coproporphyrin is usually in the range of 100-500 γ per 24-hour sample, with from 50-90% of Type III isomer, as compared with 20-100 γ and 8-35%, normally. This abnormal excretion is contrasted with the marked increase of Type I isomer previously found to characterize the urine in cases of infectious hepatitis.

15704

Experimental Evidence of the Secretion of Urine by the Fetal Kidney.*

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In the fetal rat, ligation of the ureter causes hydronephrosis and ligation of the urogenital papilla causes filling of the bladder.[1] The rate of formation of fluid by the fetal kidney is surprisingly rapid and it may be accelerated experimentally by ligating the renal pedicles of the mother or by injecting a highly concentrated solution of urea under the skin of the fetus.[2]

The present study which grew out of the experiments noted above, has been designed to determine whether this fluid is actually urine or merely a transudate of the fetal blood. In order to attain this objective, we have studied the blood and contents of the bladder of the fetus and also those of the mother.

The material for analysis was collected in the Department of Anatomy from rats in which the pregnancies were dated from the moment of observation of coitus. On the 21st day of pregnancy (20 days and 17 hours ± 2 hours), the mother was anesthetized with ether and her abdomen was opened surgically. Each fetus of a litter was transferred to the abdominal cavity of the mother[3] and its urogenital papilla was ligated by means of an unraveled strand of surgical silk. Then the abdominal incision of the mother was closed by suturing and the anesthesia was discontinued. On the 22nd day of pregnancy and about 2 hours before autopsy, the urinary papilla of the mother was ligated by means of braided silk (Champion No. 6). A few hours before the estimated time for normal parturition, the mother and her fetuses were killed by decapitation (killed at about 21½ days after coitus). Blood emerging during decapitation was allowed to drop into small vials which previously had been moistened with heparin solution. The maternal urine was secured by puncturing the bladder with a hypodermic needle (gauge 27) attached to a 10 cc syringe. The fetal urine was obtained by puncturing the bladder with a micro-pipette prepared from glass tubing and equipped with a small rubber bulb (a "policeman," commonly used in chemical laboratories).† By pooling the samples from the fetuses of one mother or, more rarely, from those of several mothers, it was possible to obtain sufficient material for the analyses.

The chemical methods employed were micro-modifications of the following standard

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* Aided by grants from the John and Mary R. Markle Foundation and from the medical research funds of the Graduate School.

[1] Wells, L. J., *Anat. Rec.*, 1946, **94**, 504.

[2] Wells, L. J., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 287.

[3] Wells, L. J., *Anat. Rec.*, 1946, **94**, 530.

† The pipettes were prepared by Dr. J. Francis Hartmann.

TABLE I.
Analysis of Fetal and Maternal Fluids.

Tests	Fetal fluids		Maternal fluids	
	No. of observations	Quantities and ratios	No. of observations	Quantities and ratios
Amt of urine in individual bladders (cc)	50	0.05-0.1*	30	(1.0-1.5)
Specific gravity of urine	9	(1.013-1.018)† 1.015‡	4	(1.026-1.030) 1.029
Blood urea N (mg/100 cc)	9	(11.7-23.0) 16.73	10	(12.1-19.0) 15.9
Urine urea N (mg/100 cc)	8	(61-384) 195.0	3	(454-556) 497.0
Ratio urine urea/blood urea	8	(3.6-23.0) 11.7	3	(29.0-35.0) 31.0
Blood creatinine (mg/100 cc)	3	(0.7-1.2) 1.0		
Urine creatinine (mg/100 cc)	1	42.0		
Ratio urine creatinine/blood creatinine	1	42.0		
Urine chlorides, as NaCl (mg/100 cc)	2	178.0	2	265.0
Apparent glucose in urine (mg/100 cc)	5	40-50	7	50-60

* In the males. (In females the bladder was usually empty since the normal hypospadias militates against blocking the urinary outlet by ligating the urinary papilla.)

† Range.

‡ Average.

methods: blood urea—incubation of blood with equal amount of urease suspension and determination of ammonia by nesslerization on Folin-Wu filtrate thereof;[4] urine urea—determination of ammonia by nesslerization before and after incubation with urease;[4] creatinine in blood;[5] creatinine in urine;[6] chlorides in urine;[7] specific gravity;[8] reducing substances in urine—Benedict's qualitative reagent with raw urine, the degree of reduction being gauged against known standards of glucose.

The suitability of these micro-modifications was established by work with pure solutions and by duplicate recoveries from the urine and blood of known small added amounts of the substances in question.

Table I gives the principal data obtained. That the fetal kidneys are functioning as such and not merely producing a transudate is shown by the ability of the kidney to concentrate certain materials and to reabsorb others.

Since the specific gravity of ultrafiltrates of mammalian plasma is approximately 1.010, the average specific gravity of 1.015 for the fluid from the fetal bladder indicates that overall concentration has occurred. Otherwise stated, this is evidence of active reabsorption of water. If the principle of Long's coefficient is applied, the indication is that there has been in the urine a 50% increase in total solids over that of the glomerular filtrate.

The approximately 12-fold concentration of urea is distinctly less than the 31-fold concentration occurring simultaneously in the mother. However, when it is recalled that the placenta is functioning as an excretory organ, definite evidence is provided that the fetal kidney is already assuming a postnatal role. In this connection, it may be pointed out that the average blood urea nitrogen (b.u.n.) of the fetuses was 0.83 mg% lower than that of their mothers. Had the b.u.n. of the fetus been higher than that of the mother, it might have been taken as evidence of beginning separation of the placenta and such elevation of the fetal b.u.n. might have been considered as a possible stimulus to the fetal kidney. However, a definite elevation of fetal over maternal b.u.n. was observed in only one of 8 observations (10.6 mg%).

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[4] Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry*, Williams and Wilkins, Baltimore, 1932, Vol. 2, vii + 957 pp.

[5] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, **38**, 81.

[6] Folin, O., *J. Biol. Chem.*, 1914, **17**, 469.

[7] Schales, O., and Schales, S. S., *J. Biol. Chem.*, 1941, **140**, 879.

[8] Barbour, H. G., and Hamilton, W. F., *J. Biol. Chem.*, 1926, **69**, 625.

Because of the known back diffusion of urea through the tubules a more active clearance of creatinine than of urea might be expected. This proved to be the case.

In addition to these evidences of selective concentration there are the evidences of selective resorption. The exact amount of glucose in the urine was not determinable. However the reducing power of the fetal urine was less than that of the corresponding maternal urine. (After yeast fermentation, reducing substances were no longer detectable in either urine by the method employed). In the light of the concentration of other substances, it can be safely concluded that active resorption of glucose occurred. In this connection it is of interest that histochemical stains show alkaline phosphatase‡ to be already clearly demonstrable in the proximal portion of the secretory tubules of these fetal kidneys.[9]

The resorption of chloride is shown to be more active in the fetus than in the mother.

In further consideration of our observations, the survival of fetal rats taken by Caesarian section indicates that the kidneys are able to function before term,[9] as in premature infants. But it is a different problem as to whether they actually function during the period when the placental circulation is still intact. The conditions of our experiments were such that the urine we obtained from the bladder of the fetus must have been produced exclusively during this period. This suggests that fetal urine is the fluid which distends the urinary passages of stillborn infants with hydronephrosis.[10,11] Makepeace and collaborators[12] and Jacqué[13] have analyzed fluid from the bladder of fetuses. Although these investigators failed to make it clear that all of the fluid they analyzed was actually produced before the placental circulation had been interrupted, their papers have internal evidence that this was the case.

Our observations indicate that in fetal rats the urine is more or less dilute. To this extent, our findings are in complete accord with those of other investigators.[12,13] That the fetal urine is actually dilute might be expected in view of the fact that the kidneys of infants are not as effective as those of adults.[14]

It is perfectly clear that the work performed by the fetal kidney is not essential for the survival of the fetus and that the placenta (hemochorial) is the essential excretory organ until birth. Recently Potter[15] has reported a series of 20 infants with bilateral agenesis of the kidney.§

Finally, the question arises as to whether the production of urine by the human fetus is a factor in such clinical disorders as hydramnios. An experimental approach to this problem, using the rat, is in progress at the University of Minnesota.

Summary. The bladders of fetal rats were allowed to fill after the urogenital papilla had been ligated. The contents of the bladder and the blood of the fetus were analyzed, as were also those of the mother. The observations indicate that the fetal kidney produces urine which is somewhat dilute.

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‡ In fetuses in which hydronephrosis is produced experimentally by ligating the ureter, the phosphatase in their kidneys is being studied by histochemical and microchemical methods.

[9] Wells, L. J., unpublished observations.

[10] Wells, L. J., and Bell, E. T., *Arch. Path.*, 1946, **42**, 274.

[11] Dohrn, *Monatschr. f. Kinderheilkunde*, 1881, **2**, 98.

[12] Makepeace, A. W., Fremont-Smith, F., Dailey, M. E., and Carroll, M. P., *Surg., Gynec. and Obstet.*, 1931, **53**, 635.

[13] Jacqué, L., *Arch. Internat. Physiol.*, 1906, **3**, 463.

[14] Young, W. F., McCance, R. A., and Dobbs, R., *Proc. Roy. Soc. Med.*, 1943, **36**, 219.

[15] Potter, E. L., *J. Pediat.*, 1946, **29**, 68.

§ In such non-placental forms as chick embryos the nitrogenous wastes are eliminated by the mesonephros and deposited in the allantois.[16,17] During the first 16 days of incubation, the allantois is the only "urinary bladder."[17]

[16] Boyden, E. A., *J. Exp. Zool.*, 1924, **40**, 437.

[17] Fiske, C. H., and Boyden, E. A., *J. Biol. Chem.*, 1926, **70**, 535.

15705

Role of Hemoconcentration in Production of Gastric and Duodenal Ulcer Following Experimental Burns.*

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The occurrence of hemoconcentration following extensive burns in man, first noted by Baraduc,[1] is well known. Hemoconcentration was suggested by Kapsinow as the mechanism of the production of Curling's Ulcer.[2] The role of sepsis in the mechanism of the occurrence of gastric and/or duodenal ulceration following experimental burns has been studied by Hartman.[3]

The purpose of this study is (1) to evaluate the role of hemoconcentration in the production of ulceration of the gastro-intestinal tract following burns, and (2) to correlate the incidence of hemoconcentration to the incidence of ulceration in dogs subjected to extensive superficial burns, with and without accompanying administration of histamine-in-beeswax mixture, with and without treatment directed to the prevention of hemoconcentration.

When daily intra-muscular injections of histamine-in-beeswax mixture are given to normal dogs, 40 days (or an average of 23 days) are necessary to produce ulcer with regularity.[4]

Method. Healthy dogs, weighing 15 to 36 pounds, anesthetized by intravenous injection of sodium pentobarbital (15 mg per pound), were subjected to 40%, third degree burns by immersion into water at 100°C for 10 to 15 seconds. No evidence of pain or discomfort to the animal was noted. The hair was closely clipped prior to scalding. Blood studies were carried out on all dogs, 3 determinations before the burn for the average of normal values, and serial determinations after the burn. The following determinations were made: Hemoglobin (alkaline hematin method), Hematocrit (Wintrobe method), Erythrocyte Count, Specific Gravity of whole blood and plasma (copper sulfate method of Phillips, Van Slyke, *et al.*), BUN (Karr's urea nitrogen method), Plasma and Blood Volumes (Gibson and Evan's method), and Rectal Temperatures. Blood for these determinations was obtained under oil from the femoral vein before the burn and from the heart following the burn. A number of the dogs received, in addition, daily intramuscular injections of histamine-in-beeswax mixture (30 mg base) prepared after the method of Code and Varco,[5] the first injection being given 30 minutes prior to the burn. No restriction of food intake was made, except that dogs were fasted for 18 hours before each experiment. Water intake was allowed up to 250 cc per day in all dogs. In some of the animals an attempt was made to prevent the hemoconcentration following burns by slow intravenous administration of human dried plasma into an antecubital vein, regulation of the rate of drip being determined by serial blood studies. Blood pressure determinations were made in 3 dogs by serial or continuous recordings on a kymograph, the carotid artery being cannulated. Dogs were sacrificed 4 hours to 6 days following the burn. Animals in Series II were sacrificed when death was impending or allowed to die. Sections of the stomach, duodenum, liver, lung, brain, kidney, and adrenals were obtained for microscopic studies.

Experiments. Studies were carried out on 30 dogs in 3 series of experiments:

Series I. Fourteen dogs were subjected to 40%, third degree burns accompanied by

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* This study is supported in part by the Dr. and Mrs. Harry B. Zimmerman Fund for Surgical Research and the Graduate School of the University of Minnesota, Minneapolis, Minn.

[1] Harkins, H. N., *The Treatment of Burns*, Springfield, Illinois, Charles C. Thomas, publisher, 1942.

[2] Kapsinow, R., *Southern Med. J.*, 1934, **27**, 500.

[3] Hartman, F. W., *Gastroenterology*, 1946, **6**, 130.

[4] Hay, L. J., Varco, R. L., Code, C. F., and Wangensteen, O. H., *Surg., Gyn. and Obst.*, 1942, **75**, 170.

[5] Code, C. F., and Varco, R. L., *Am. J. Physiol.*, 1942, **137**, 225.

TABLE I.

Dogs Subjected to 40% Burn Accompanied by Daily Administration of Histamine-in Beeswax (30 mg Base), Not Treated with Intravenous Infusion.

Dog No.	Wt in lb	Burn		No. of hist. injections	Hematocrit change		Results	Remarks
		%	sec		Initial	Final		
23	29	40	10	4	40	41	No congestion, erosion, or ulcer	Died—anorexia
24	32	40	10	5	44	55	Congestion; duodenal ulcers, one perforated	Died—peritonitis
25	22	40	10	3	42	53	Congestion; pyloric and duodenal ulcers	Died the third day
36	17	40	15	1	49	56	Congestion; small pyloric ulcer; many bleeding points	Sacrificed, 24 hr
37	22	40	15	2	43	65	Congestion; bleeding pyloric ulcer; two large duodenal ulcers	,, 48 ,,
38	24	40	15	2	50	61	Congestion; pyloric-duodenal ulcer; bleeding small fundic ulcers	Died second day—melena
39	22	40	15	2	30	41	Congestion; large duodenal ulcers, one perforated; fundic ulcers	Died—peritonitis
40	24	40	15	2	50	55	Congestion; three large duodenal ulcers; one large gastric ulcer; many small bleeding ulcers	Sacrificed—melena
42	15	40	15	1	40	57	Congestion; marked bleeding in stomach and duodenum	,, 18 hr
44	20	40	15	1	49	72	Intense congestion	,, 4 ,,
45	26	40	15	3	47	56	Congestion; bleeding antral ulcer	Died third day
50	22	40	10	1	42	42	No congestion, erosion, or ulcer	Sacrificed, 24 hr
51	36	40	10	1	49	54	Congestion only	,, 6 ,,
52	31	40	15	1	45	53	Congestion; large duodenal ulcer; many petechial gastric ulcers	,, 24 ,,

histamine-in-beeswax administration. Blood determinations were carried out in all animals.

Series II. Five dogs were subjected to 40%, third degree burns alone, blood determinations being carried out in all dogs.

Series III. Six dogs were subjected to 40%, third degree burns accompanied by histamine-in-beeswax administration, and attempts to prevent the hemoconcentration were carried out by intravenous infusion of plasma. One dog (No. 41) received, in addition, 5% glucose in normal saline, and another dog (No. 49) received 5% glucose in normal saline alone. As an additional control, one dog (No. 48), not subjected to a burn, received histamine-in-beeswax, was anesthetized, and received a comparable amount of plasma by intravenous infusion. Four additional dogs received daily intramuscular injections of histamine-in-beeswax for 5 successive days, and were sacrificed at the end of this period.

Results. Series I. (See Table I). Twelve of the 14 dogs subjected to 40% burns accompanied by histamine administration developed increased concentration of the blood. All 12 dogs demonstrated marked congestion of the mucous membrane of the stomach and duodenum. Nine of these 12 dogs were sacrificed 24 hours or more after the burn and showed definitive ulcers, 5 bleeding and 2 perforated. Three of the 12 dogs were sacrificed at 4, 6, and 18 hours after the burn, the latter showing in addition to the congestion, marked diffuse bleeding from the surface of the gastric and duodenal mucosa. The 2 dogs which failed to develop increased concentration of the blood demonstrated no gastric or duodenal pathology in one and 4 days.

Series II. (See Table II). Four of the 5 dogs subjected to 40% burns without administration of histamine-in-beeswax developed increased concentration of the blood and demonstrated congestion of the gastrointestinal mucosa in 2 to 6 days. Two of these dogs showed, in addition to congestion, bleeding petechial gastric ulcers, and antral bleeding points with hemorrhagic gastritis and duodenitis. One dog failed to develop increased concentration of the blood and demonstrated no gastric or duodenal pathology in 4 days.

TABLE II.

Dogs Subjected to 40% Burn—No Histamine Administration—Not Treated with Intravenous Infusion.

Dog No.	Wt in lb	Burn		No. of hist. injections	Hematocrit change		Results	Remarks
		%	sec		Initial	Final		
26	26	40	10	0	50	56	Bleeding petechial gastric ulcers; congestion	Sacrificed 6th day
27	31	40	10	0	45	57	Hemorrhagic gastritis duodenitis; antral bleeding pts. Marked congestion	Died 2nd day
28	15	40	10	0	59	60	Essentially negative	,, 4th ,,
29	24	40	15	0	48	59	Congestion only	Sacrificed 5th day
30	30	40	15	0	45	56	,, ,,	Died 5th day

TABLE III.

Dogs Subjected to 40% Burn, Accompanied by Histamine-in-Beeswax Administration, Treated by Intravenous Infusion.

Dog No.	Wt in lb	Burn		No. of hist. injections	Concentration of blood	Results	Remarks
		%	sec				
43	21	40	15	1	Maintained at normal values	No congestion, erosion, or ulcer	Sacrificed after 18 hr
49	32	40	15	1	" " " "	" " " "	" " 24 "
53	35	40	15	1	" " " "	" " " "	" " 24 "
46	18	40	15	2	Normal except for 18 hr of hemoconcentration	Congestion, lesser curvature ulcers	" " 54 "
47	19	40	15	2	Normal except for 18 hr of hemoconcentration	Congestion, perforated duodenal ulcers	Died, peritonitis, 54 hr
41	22	40	15	1	Overtreated—hemodilution	Pallor, submucosal and mucosal hemorrhage and erosion of stomach	Died 16 hr

Series III. (See Table III). Of the 6 dogs subjected to 40% burns accompanied by histamine-in-beeswax administration in which an attempt at prevention of the hemoconcentration in burns was made, 3 dogs were well controlled throughout the entire duration of the experiment; 2 dogs, partially controlled; and one dog, overtreated. The 3 dogs in which blood concentration values were maintained at normal levels demonstrated no gastric or duodenal pathology when sacrificed in 18 to 24 hours. The 2 dogs in which normal blood concentration values were maintained except for a period of 18 hours out of a total duration of 54 hours, demonstrated gastro-intestinal congestion, with lesser curvature ulcers and perforated duodenal ulcer, respectively. One dog was overtreated in the first few hours of the experiment so that marked hemodilution occurred. Death occurred in this animal in 16 hours, presenting pallor of the stomach with several areas of mucosal and submucosal hemorrhage and erosions.

Microscopic examination of the viscera of the animals developing hemoconcentration showed marked vascular congestion; of the animals in which hemoconcentration did not take place or was prevented, no significant findings were noted.

Four dogs receiving daily intramuscular injections of histamine-in-beeswax for 5 days developed no hemoconcentration and no gastro-intestinal abnormality.

Discussion. It is apparent that there exists a close correlation between increased concentration of the blood and gastro-intestinal abnormality following burns. That hemoconcentration, with its resultant congestion, renders the gastro-duodenal mucosa more susceptible to the action of gastric acid-peptic juice is evident in the marked increase in the incidence of ulceration when histamine-in-beeswax administration accompanies the burn. It is shown, in addition, that prevention of the occurrence of hemoconcentration following severe burns portends a normal gastro-intestinal tract, even when histamine-in-beeswax is administered and animals are sacrificed in less than 5 days. Failure of 3 dogs in Series I and II to develop hemoconcentration and mucosal pathology following the burns may be attributable to the fact that those animals received a 40% 10-second burn, a less severe burn than the majority of the animals in this study received. Serial blood pressure determinations were made in 3 burned dogs, continuously anesthetized, which developed hemoconcentration, and no readings of shock level were observed. Animals were usually awake and moving about after 4 hours following the burn.

It is interesting to note that superficial necrosis of the gastric mucosa was present upon microscopic examination in 2 dogs, both sacrificed, one after marked hemodilution due to overtreatment, the other after marked hemoconcentration and congestion of the mucosa. Microscopically, the vascular spaces of the mucosa of the overtreated animal were conspicuously devoid of blood, while those of the untreated animal were markedly dilated and engorged with blood with extravasation and necrosis. Both conditions result in mucosal ischemia, susceptible to the erosive action of gastric acid-peptic secretions.

Conclusions. Evidence is presented to show that increased concentration of the blood following burns is an important factor in the occurrence of gastro-duodenal ulcer after experimental burns. (The occurrence of gastro-intestinal congestion, erosion and/or ulcer in burns is directly related to the occurrence of hemoconcentration).

Moreover, gastro-intestinal abnormality following burns, even when accompanied by histamine administration, may be prevented by avoidance of the hemoconcentration of burns by proper therapy.

The incidence of gastric and/or duodenal ulceration provoked by hemoconcentration in burns is markedly increased when histamine-in-beeswax administration accompanies the burn.

15706

Comparative Utilization of Raw and Autoclaved Soy Bean Protein by the Human.

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(With the technical assistance of M. A. Adams and L. J. Harrison.)

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Osborne and Mendel[1] in 1917 noted that rats fed raw soy bean flour grew more slowly than a control group which received the same flour after it had been autoclaved. Hayward, Steenbock and Bohstedt[2] confirmed these investigations and suggested that this effect was not due to a difference in palatability but to a deficiency in the raw flour.[7] Later investigations by Hayward and Hafner,[3] Almquist, Mecchi, Kratzer and Grau[4] and Johnson, Parsons, Steenboch[5] suggested that this increase in biological activity produced by autoclaving raw soy bean flour was due to a rise in the level or increase in the availability of sulfur bearing amino acids.

That the autoclaved soy bean protein has a high biological value similar to egg white and liver protein has been shown by Cahill, Schroeder and Smith[6] from a study of ni-

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[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, **32**, 369.

[2] Hayward, J. W., Steenbock, H., and Bohstedt, G., *J. Nutrition*, 1936, **11**, 219.

[3] Hayward, J. W., and Hafner, F. H., *Poultry Science*, 1941, **20**, 139.

[4] Almquist, H. J., Mecchi, E., Kratzer, F. H., and Grau, C. R., *J. Nutrition*, 1936, **63**, 277.

[5] Johnson, Y. M., Parsons, H. T., and Steenboch, H., *J. Nutrition*, 1939, **18**, 423.

[6] Cahill, W. M., Schroeder, L. J., and Smith, A. H., *J. Nutrition*, 1944, **28**, 209.

[7] McNaught, J. B., Scott, V. C., Woods, F. M., and Whipple, G. H., *J. Exp. Med.*, 1936, **63**, 277.

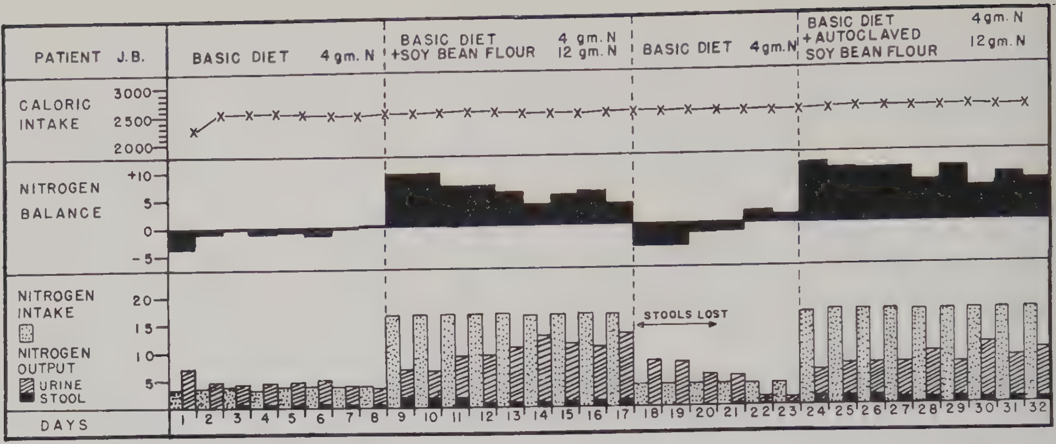

FIG. 1.
Nitrogen balance in Patient J. B.

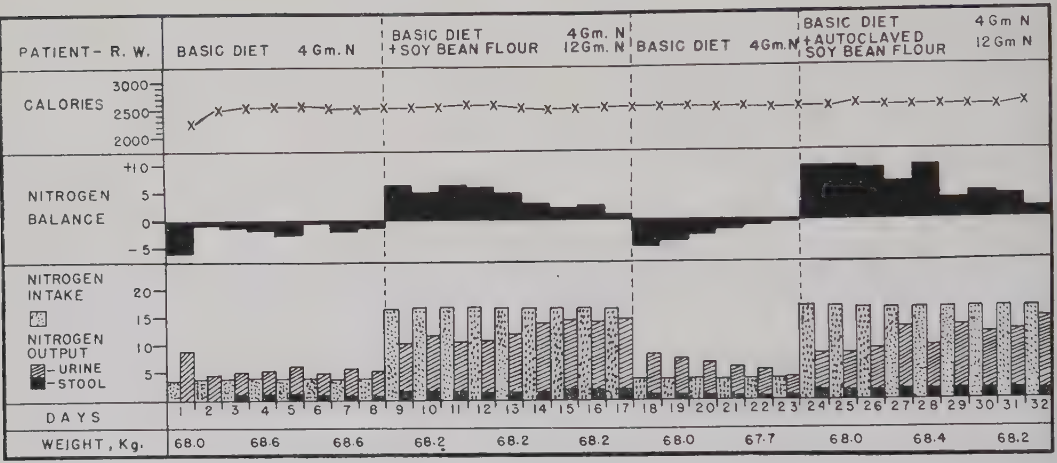

FIG. 2.
Nitrogen balance in Patient R. W.

trogen balance in humans and by McNaught, Scott, Woods and Whipple[7] from a study of plasma protein regeneration in dogs.

Recently another explanation has been offered for the apparent lower biological activity of raw soy bean protein. Ham and Sandstedt[8] isolated a trypsin-inhibiting substance from the unheated soy bean flour which they felt was identical with its growth-retarding action. In later studies, Ham, Sandstedt and Musseh[9] showed that this trypsin-inhibiting substance isolated from soy bean flour had a retarding effect upon the normal growth of chicks; and, by *in vitro* test they showed that the proteolytic activity in the small intestine of the chick was greatly inhibited. Similar results were obtained by Klose, Hill and Fevold[10] in growing rats.

Bowman[11] demonstrated the inhibition of the tryptic digestion of casein by a fraction of an aqueous extract of raw soy bean. This was also shown by Kunitz[12] who later isolated this trypsin inhibitor in crystalline form.[13]

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[8] Ham, W. E., and Sandstedt, R. M., *J. Biol. Chem.*, 1944, **154**, 505.

[9] Ham, W. E., Sandstedt, R. M., and Mussehl, F. E., *J. Biol. Chem.*, 1945, **161**, 635.

[10] Klose, A. A., Hill, B., and Fevold, H. L., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 10.

[11] Bowman, D. E., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 139.

[12] Kunitz, M., *Science*, 1945, **101**, 668.

[13] Kunitz, M., *J. Gen. Physiol.*, 1946, **29**, 149.

Tagnon and Soulier[14] have shown that this trypsin inhibitor acts *in vitro* as an anticoagulant of whole blood and plasma, suggesting that it may also inhibit the plasma enzyme associated with blood coagulation.

In view of the current widespread use of soy bean flour as a human food, and because of the observations made in animals, the investigations reported here were undertaken to compare the utilization of raw and autoclaved soy bean flour in humans.

Methods and Materials. Two cooperative, well nourished patients, one with a diagnosis of mild osteoarthritis; the other a hemophiliac without hemorrhagic manifestations, were chosen as subjects. Both patients were allowed unrestricted activity about the ward and their caloric requirements were estimated at 2500 calories. The basic diet was, therefore, planned to afford 2500 calories daily and provided a low protein intake of 25 g from mixed sources, about 75% of which was of vegetable origin. After an initial control period of 8 days, during which the subjects received only the basic diet, 188 g of raw soy bean flour containing 75 g of protein was substituted for an equal caloric value of carbohydrate in the basic diet. The soy bean flour was given as a liquid supplement dissolved in water to a volume of one liter. After this 9-day period on raw soy bean flour the subjects returned to the basic diet for 6 days. This was followed by another 9-day trial period during which the subjects received 188 g of soy bean flour previously autoclaved for one hour at 15 lb pressure.

Nitrogen balance and body weights were followed throughout the study period.

Urine nitrogen determinations were done by micro-Kjeldahl method on aliquot of 24-hour specimens, and stool nitrogens were determined by macro-Kjeldahl method on 3-day pooled specimens.

Results. Fig. 1 and 2 show the caloric intake and nitrogen balances of these 2 patients. The average daily nitrogen retention of patient J.B. was 6.7 g on raw soy bean flour and 8.5 g on the autoclaved material. Patient R.W. showed similar results with an average daily nitrogen retention for the first period of 4.1 g and for the second period of 5.4 g. In each of these subjects the nitrogen retention is about 20% greater when the autoclaved material is used instead of the raw material. Increase in stool nitrogen does not account for this difference as subject J.B. showed an average daily stool nitrogen of 1.3 g during the first period and 1.1 g during the second period, while subject R.W. showed 1.6 g in the first period and 1.8 g in the second period. There was no significant change in body weight in either patient.

The soy bean supplements were not disagreeable in taste. Both patients complained of flatulence during both of the trial periods. There were no other gastro-intestinal symptoms.

Discussion. Both the raw and autoclaved soy bean protein support positive nitrogen balance in adult humans. Retention of nitrogen is about 20% greater during the administration of the autoclaved material than with the raw material. The cause of this greater biological activity is not clear from these investigations. There is no increase in excretion of nitrogen in the stool following intake of raw material, suggesting that intestinal protein digestion has progressed far enough to produce absorbable nitrogen products. Whether all these products absorbed from nonautoclaved soy bean flour are capable of entering the metabolic pool requires further study.

Conclusions. 1. Both raw and autoclaved soy bean protein support positive nitrogen balance in the adult human. 2. Nitrogen retention is about 20% greater with the autoclaved material than with the raw material.

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[14] Tagnon, H. J., and Soulier, J. P., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 440.

15707

Susceptibility of the Bear to Fox Encephalitis.

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Fox encephalitis[1] is a common disease of the North American silver fox and has been identified on fox ranches throughout the United States. Red foxes, dogs, and coyotes[2] are also highly susceptible. Although not generally recognized clinically, fox encephalitis frequently appears to occur as a natural disease in dogs.[3] The gray fox,[4] which is related to the South American foxes and dogs, and the raccoon[5] are only slightly susceptible to experimental infection.

Inoculation of the anterior chamber of the eye affords a direct and easy method of identifying the fox encephalitis virus and of establishing susceptibility to infection.[6] In foxes and dogs such inoculation results in an acute systemic infection and encephalitis with a high mortality. In the inoculated eye the virus damages the single layer of endothelial cells which line the posterior surface of the cornea, resulting in a grayish corneal opacity. The characteristic intranuclear inclusions produced by the virus can be seen in these endothelial cells. Only the injected eye will show the opacity. Injection of the virus into the anterior chambers of the eyes of the less susceptible raccoon and gray fox produces regularly the corneal opacity, but these animals otherwise generally remain well. The cat appears immune to the general infection, but with young kittens[7] intra-ocular injection either is negative or results in incomplete opacity. The eye inoculation has been found negative in rabbits.[7]

We have now been able to show that the zoologic range covered by the virus of fox encephalitis also includes the black bear, *Euarctos americanus*. Three specimens of this animal received at our laboratories have been tested for susceptibility to the virus.

Fox encephalitis virus, preserved as 20% homogenized fox brain in 50% neutral glycerin, was prepared in serial, ten-fold dilutions in Ringer's solution. To determine the minimal infective dose that would produce corneal opacity in foxes, 0.1 cc of each dilution was injected into the anterior chambers of the eyes of red fox pups. Since we desired only to approximate the titer of the virus, only one eye was inoculated per dilution, and the dilutions were carried out to 10^{-9}. The virus proved active at a dilution of 10^{-6}.

A young, wild black bear was received in the laboratory in rather poor condition. It was underweight, extremely irritable, and did not eat well. This animal was anesthetized with ether and was injected intra-ocularly with 0.1 cc of a 10^{-2} dilution of virus in the right eye and 0.1 cc of a 10^{-4} dilution in the left eye. Within 3 days the bear developed a complete opacity in its right eye and a partial opacity in its left eye. On the 4th day after inoculation the animal was found dead, without previous symptoms or autopsy findings of fox encephalitis. However, a smear of the endothelial cells of the posterior surface of the cornea revealed the characteristic fox encephalitis inclusion bodies. Aqueous humor removed from the eyes was cultured on blood agar and was found free of bacteria.

The intra-ocular test was repeated on a second young black bear. This animal although somewhat underweight otherwise appeared normal. The virus, diluted with saline, was inoculated into the anterior chambers of the left and right eyes, with 0.2 cc of 10^{-2} and 10^{-4} dilutions respectively as the inoculum. This animal showed no sign of susceptibility to the virus until the 10th day after inoculation, when partial corneal opacity of the left eye appeared, becoming complete 2 days later. No further observations were possible since it became necessary at that time to kill the bear. However, the animal did exhibit intranuclear inclusions of fox encephalitis virus in the corneal endothelium. The aqueous humor was bacteriologically sterile.

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[2] Green, R. G., Ziegler, N. R., Carlson, W. E., Shillinger, J. E., Tyler, S. H., and Dewey, E. T., *Am. J. Hyg.*, 1934, **19**, 343.

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[6] Evans, C. A., Yanamura, H. Y., and Green, R. G., Proc. Soc. Exp. Biol. and Med., 1943, **53**, 183.

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A third black bear, which was half grown, was inoculated intramuscularly with 5 cc of 20% virus but did not develop observable symptoms.

Conclusions. The intra-ocular inoculation of two bears and the intramuscular injection of a third with fox encephalitis virus indicate that the black bear *(Euarctos americanus)* is only slightly susceptible to this virus infection. Since the bear, like the raccoon, is an offshoot of the canines, the pathogenic properties of fox encephalitis virus derived from foxes seem sharply confined to the canine family.

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Two Related Salmonella Types: *S. luciana* and *S. marseille*.*

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A. *Salmonella luciana* was isolated by Mrs. Mildred Galton from the feces of a normal food handler. The organism fermented glucose, xylose, arabinose, rhamnose, maltose, cellobiose, trehalose, mannitol, sorbitol, and dulcitol with production of acid and gas. Lactose, sucrose, inositol, and salicin were not fermented. The culture utilized *d, l,* and *i*-tartrate, mucate, and citrate. Hydrogen sulfide was produced but indol was not formed nor was gelatin liquefied.

The organism was agglutinated to the titre of, and absorbed all agglutinins from *S. aberdeen* O serum. Its somatic antigens are XI.

S. luciana was diphasic and the H antigens of phase I were identical with the H antigens of *S. paratyphi A* (a). The antigens of phase 2 were identical with phase 2 of *S. glostrup* (e,n,z_{15} . . .). The antigenic formula of *S. luciana* is XI:a-e,n,z_{15} . . .

B. *Salmonella marseille* was isolated from the stools of a person affected with gastro-enteritis, by Captain W. B. Sutton of the 4th Medical Laboratory, U. S. Army. Its biochemical properties differed from those of *S. luciana* only in that it produced acid from raffinose after 9 days incubation and it did not utilize *i*-tartrate.

The somatic antigens of *S. marseille* were identical with those of *S. aberdeen* (XI). The culture was diphasic and phase 1 resembled that of *S. paratyphi* A (a) although when used to absorb *S. paratyphi A* serum a slight residue of agglutinins remained for *S. paratyphi A, S. luciana,* and *S. loma-linda.*

Phase 2 of *S. marseille* was agglutinated by serums for all the nonspecific phases of the Kauffmann-White classification. When tested with serums for single factors 2, 3, 5, 6, 7, 10, and 11 it was agglutinated only by serums for factors 3 and 5. In absorption tests phase 2 of *S. marseille* removed all agglutinins from serum derived from phase 2 of *S. thompson* (1,5 . . .). The diagnostic formula of *S. marseille* is XI:a-1,5 . . .

Summary. Two new Salmonella types were described. *S. luciana* (XI:a-e,n,z_{15} . . .) was recovered from the feces of a normal food handler while *S. marseille* (XI:a-1,5 . . .) was isolated from the stools of a patient affected with gastro-enteritis.

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* The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director. It was supported in part by a research grant from the U. S. Public Health Service.

15709

Plasma Protein Concentrations and Organ Weights of Rats as Related to a High Protein Diet.*

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Adrenal hypertrophy resulting in an increased functional level of the cortex has been observed in rats fed a high protein diet and may be related to an increased rate of protein catabolism[1] but not all investigators have observed an increase in adrenal weight in either rats[2,3] or mice[4] eating a diet high in protein content. Furthermore, no change in plasma protein concentrations was observed in rats fed a 78% casein diet.[3] It was presumed that a change in functional activity of the adrenal cortex would be reflected in plasma protein levels since an important relationship between the adrenal cortex and plasma protein concentrations is known to exist.[5,6]

Ingle[7] reinvestigated the high protein diet-adrenal weight problem in both young and adult rats fed a diet of 67% protein and found the adrenals to be unchanged, but did observe a modest adrenal weight increase when dietary protein was increased to 80%. The primary protein sources used by Ingle were casein (Labco) and lactalbumin. Since a difference in the protein source itself can influence the experimental results as has been effectively demonstrated in adrenalectomized rats given 2 types of casein,[8] the current investigation involving a restudy of the adrenals in rats fed a high protein diet was undertaken and plasma protein levels were again used as a possible means of detecting an increased functional activity of the adrenal cortex.

Adult male rats 130 to 157 days of age were used. The diets fed were Purina fox chow as the control diet (22.8% protein) and the high protein diets which were composed of 78% casein (Merck) or lactalbumin (Borden, lot No. 15-42), 10% brewers yeast (Mead-Johnson), 5% Wesson's salt mixture, 5% Mazola and 2% cod liver oil (Squibb). The diet including casein simulated the Casein A diet of Tepperman *et al.*[1] Lactalbumin was chosen as another primary protein source because the nitrogen balance index for this protein is higher than that of casein.[9]

The experimental period was 20-27 days in duration and during the first 20 days food intake ranged between 280-291 g total per rat fed the high protein diets as compared with a 336 g total per rat fed fox chow. The rats were bled by cardiac puncture under light anesthesia at the conclusion of the experiment and the plasma analyzed for NPN, total protein, albumin and globulin by methods reported previously.[10] Each rat was autopsied and fresh weight of the carefully dissected pituitary, adrenal, thyroid, testis, seminal vesicle, kidney and liver was recorded.

Adult rats fed a high casein diet gained an average of 16 g in body weight as compared with a 28 g gain by rats eating fox chow. When lactalbumin was substituted for casein an average gain of only 4 g in body weight was recorded whereas the littermate controls

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* Supported by the Protein Metabolism Fund of the Bureau of Biological Research, Rutgers University.

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TABLE I.
Influence of Diet on Organ Weight of Adult Male Rats.

Diet (No. of rats)	Casein 78% (14)	Fox chow (14)	Lactalbumin 78% (12)	Fox chow (12)
	Avg organ weight—mg.			
Adrenal	34.8 ± 2.2*	28.4 ± 1.4	34.8 ± 2.2	28.6 ± 2.0
Thyroid	23.6 ± 0.7	23.0 ± 0.5	22.8 ± 0.9	22.6 ± 0.4
Kidney	3248.0 ± 49	2652.0 ± 47	2962.0 ± 123	2811.0 ± 157
Liver	13579.0 ± 289	12303.0 ± 302	12595.0 ± 655	12649.0 ± 341
	Avg organ weight—mg/100 g body weight.			
Adrenal	10.7 ± 0.7	8.7 ± 0.5	11.2 ± 0.7	8.8 ± 0.6
Thyroid	7.3 ± 0.2	7.0 ± 0.2	7.4 ± 0.3	7.3 ± 0.1
Kidney	1008.0 ± 15	818.0 ± 14	959.0 ± 42	787.0 ± 57
Liver	4192.0 ± 83	3786.0 ± 94	4009.0 ± 220	3870.0 ± 97

$$^{*}\epsilon = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

TABLE II.
Influence of Dietary Protein on the Plasma Protein Levels of Male Rats.

No. of rats	Diet	Body wt start-end, g	Hemato-crit, %	Non-protein N, mg/100 cc	Total protein, g/100 cc	Albumin, g/100 cc	Globulin, g/100 cc
			Rats 130-157 days old.				
14	Casein 78%	307-323	48.5 ± 0.7	76 ± 1.8	6.08 ± 0.15	3.06 ± 0.9	3.02 ± 0.08
14	Fox chow	296-324	47.6 ± 0.6	64 ± 2.0	6.13 ± 0.11	3.16 ± 0.10	2.96 ± 0.10
12	Lactalbumin 78%	309-313	47.5 ± 0.7	73 ± 2.1	6.01 ± 0.14	3.06 ± 0.14	2.95 ± 0.16
12	Fox chow	307-326	47.4 ± 0.3	60 ± 1.1	6.23 ± 0.12	3.29 ± 0.10	2.97 ± 0.16
			Rats 45-49 days old.				
8	Lactalbumin 78%	142-204	43.3 ± 0.5	80 ± 2.0	5.40 ± 0.19	3.20 ± 0.10	2.20 ± 0.17
8	Fox chow	144-227	44.4 ± 0.4	51 ± 1.2	5.87 ± 0.18	3.45 ± 0.09	2.42 ± 0.20

TABLE III.
Influence of Diet on Organ Weight of Young Male Rats.

Diet (No. of rats)	Lactalbumin 78% (8)	Fox chow (8)	Lactalbumin 78% (8)	Fox chow (8)
	Avg organ wt, mg		Avg organ wt, mg/100 g B.W.	
Adrenal	33.9 ± 3.2	27.2 ± 2.3	15.8 ± 0.9	12.1 ± 0.8
Thyroid	17.2 ± 2.1	16.4 ± 1.4	8.3 ± 0.7	7.5 ± 0.6
Kidney	2412.0 ± 161	1987.0 ± 63	1161.0 ± 74	875.0 ± 30
Liver	10595.0 ± 663	9770.0 ± 538	5127.0 ± 340	4315.0 ± 261

on stock diet gained 19 g during the same 20-day period.

It is apparent from the data in Table I that the adrenals from rats fed a high protein diet of either casein or lactalbumin had a tendency to be somewhat heavier than the adrenals from rats fed fox chow but the differences are not striking. Kidney and liver weights too were greater in rats fed 78% casein but not in those fed 78% lactalbumin. This seeming difference in results between the 2 high protein diets and kidney weight is shown to be due to body weight differences (Table I). Although it has been reported that thyroid hypertrophy can be induced by excess dietary protein, no indication of a change in thyroid weight was observed. Weights of the pituitary, testis and seminal vesicles were not influenced by diet.

Plasma protein concentrations failed to reveal any evidence that the functional level of the adrenal cortex was altered by a diet containing 78% casein or lactalbumin. The data in Table II reveal that albumin and globulin concentrations and consequently total plasma protein levels were not significantly influenced by the diet. Non-protein-nitrogen, however, was elevated when excess protein was fed.

The possibility that young rats might exhibit a more pronounced response to a diet containing 78% lactalbumin than did the adults prompted an investigation of this point. Eight rats, 45-49 days old, were fed the 78% lactalbumin diet for an average of 23 days and the data were compared with littermate controls fed fox chow. Despite *ad libitum* feeding, the rats consuming the high protein diet gained an average of only 62 g as compared to the average 83 gain exhibited by rats eating fox chow (Table II). Despite the retardation in body weight gain, the adrenal, kidney and liver weights from rats fed a 78% lactalbumin diet exceeded those from rats fed stock diet. These organ weight differences are more apparent when organ weight is considered in terms of 100 g body weight (Table III). The weight of the thyroid, testis, seminal vesicles and pituitary was not influenced.

Examination of the plasma protein concentrations for total protein, albumin and globulin suggested that rats fed fox chow had the higher plasma protein levels but the differences are not clearly significant. NPN, however, was significantly higher in rats fed the high protein diet.

Since liver protein can be readily influenced by a dietary deficiency in protein it was of interest to examine the livers from some of these young rats under the opposite conditions or that of protein excess. The livers from 4 pairs of rats were dried to constant weight at 95°C and analyzed for total protein. Water content of the livers from rats fed 78% lactalbumin was 69.9% and from rats fed fox chow was 70.0%. Liver protein was found to be greater in the lactalbumin-fed rats than in stock-diet animals. Following the feeding of the high protein diet the livers contained an average 1.15 (0.98-1.26) g protein/100 g body weight as compared with an average 0.83 (0.76-0.89) g protein/100 g body weight. Furthermore, the percentage protein of the dry liver increased from 64.0% in rats fed fox chow to 72.3% in rats fed the high lactalbumin diet and therefore the increased liver protein was not alone due to increase in liver size.

Summary. The effect of a high protein diet (78% casein or lactalbumin) was studied in male rats. The rate of body weight increase was retarded over a 3-week period by a 78% lactalbumin diet in young and adult rats. A modest increase in adrenal weight was observed but no indication of increased functional activity was suggested by alterations in plasma albumin or globulin concentrations since the plasma protein levels remained unchanged. Non-protein nitrogen was elevated by the excess dietary protein. Thyroid, pituitary, testis and seminal vesicle weights remained normal but kidney and liver weights were increased. Liver protein was increased in young rats fed the high lactalbumin diet.

15710

Pyribenzamine Aerosol Inhalation and its Influence on Histamine Poisoning and Anaphylaxis.

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From Ciba Pharmaceutical Products, Inc., Summit, N.J.

The lethal dose of histamine for guinea pigs by intravenous injection is 0.3 to 0.4 mg/kg body weight,[1] but the amounts required to kill guinea pigs when introduced into the lungs by aerosol inhalation are fractions of these doses.[2,3] Indeed, many guinea pigs breathing air containing less than 0.1 γ histamine per ml die within 2 to 5 minutes. The high activity of histamine introduced directly into the lungs is explained by two facts, namely, that the lungs of guinea pigs contain tissues whose cells are exceedingly susceptible to this substance, and that the marked response of these cells in histamine poisoning is the primary cause of death.

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Substances such as Pyribenzamine (PBZ) for example, which specifically counteract histamine, are believed to prevent histamine from acting upon organs susceptible to histamine. According to a theory accepted by many investigators, these antihistaminic substances compete with histamine, blocking the receptive sites within the susceptible cells which normally respond readily to histamine. If this theory is correct it would be expected that a specific antihistaminic substance, like PBZ, introduced directly into the lungs by aerosol inhalation would provide considerably more protection against subsequent histamine poisoning than when injected or given by mouth. Moreover, it might also be found that PBZ, as an aerosol, would counteract histamine or protect against anaphylaxis in such sufficiently small doses that side effects, common to the usual therapeutic administration, could be avoided.

Experiments with PBZ aerosol could therefore provide us with valuable information concerning the mechanism of action of antihistaminic substances. On the other hand, it seemed to us probable that a comparison of the effect of PBZ aerosols upon histamine poisoning and anaphylaxis might lead to further insight into the relationship between histamine poisoning and anaphylaxis. This question is especially important since there are many authors who consider histamine to be identical with the anaphylactic poison, while others reject the histamine theory of anaphylaxis.

In the following experiments we therefore investigated in guinea pigs the influence of PBZ aerosols upon the tolerance toward histamine and upon active and passive anaphylaxis.

Methods. 173 guinea pigs weighing from 150 to 200 g were subjected to inhalation of PBZ aerosols in the same manner as previously described for histamine aerosols.[2-6] In the various experiments performed, we vaporized solutions containing 0.5, 1, and 2% PBZ (hydrochloride), the air flow at the outlet of the vaporizers being maintained at approximately 20 liters per minute.

The influence of the aerosol inhalation upon histamine intoxication and anaphylaxis was tested as follows: In the first series of experiments, the duration of the PBZ aerosol treatment was varied and the influence of each time-treatment upon the resistance of the animals to histamine was measured. In the second series we kept the duration of aerosol-treatment constant and determined the duration of protection to histamine. In both series histamine was injected intracardially as histamine phosphate. In the third and fourth series of experiments we tested in an identical way the influence of PBZ aerosol inhalation upon active and passive anaphylaxis. Active anaphylaxis was produced with horse serum by the usual technic, the intracardial "challenge" injections being made three weeks after sensitization, and passive sensitization by injection with anti-horse rabbit serum, followed by the "challenge" injection 24 hours later.

Results. Histamine Shock. I. *Histamine Intoxication.* In preliminary trials, the sensitivity of guinea pigs to the histamine used for these experiments was determined. The minimal toxic dose producing more than 50% deaths was found to be between 0.7 to 0.8 mg histamine phosphate per kg, corresponding to 0.25 to 0.29 mg of histamine respectively. The MLD_{50} was about 25% higher in the present experiments than in our earlier work,[3] a fact which may have been due to variation among the histamine lots commercially available, differences in animal strains, minor variations in rates of injection, or to seasonal changes in the sensitivity of the guinea pigs.

II. *Influence of the Concentration and Duration of Pyribenzamine Aerosol Inhalation.*

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Inhalation of aerosols produced by vaporizing 0.5% and 1.0% PBZ solutions for various periods of time conferred definite protection against histamine to a few animals. A number of animals, however, did not show any significant beneficial response when treated with these relatively low concentrations.

Consistent results were obtained in almost all animals when the concentration of PBZ in the vaporizing fluid was increased to 2%. 34 animals were submitted to inhalations of 2% PBZ aerosol of varying duration. The shortest time of inhalation producing a definite protective effect against 2-3 lethal doses of histamine was between 1 and 2 minutes. With 2 minutes of inhalation, the animals tolerated intracardial injection of 2 mg histamine phosphate per kg, constituting approximately 2.5 lethal doses, without any symptoms of shock. With 3 minutes of inhalation they tolerated approximately 3.75 mg histamine phosphate (5 MLD), while inhalation for 5 to 10 minutes produced a resistance to 5-11 mg histamine phosphate (7 to 15 MLD).

The guinea pigs so protected did not go into shock and survived; however, all animals which were protected from typical histamine shock and had received histamine doses ranging from 5 to 11 mg per kg body weight presented very unusual signs of histamine intoxication. A few minutes after histamine injection the animals became motionless and then rapidly fell into deep narcosis with regular respiration, although very superficial and slow; there were also occasional athetotic movements of all legs. This interesting form of histamine intoxication is not normally observed, since death occurs from bronchoconstriction and asphyxia before narcosis could develop.

III. *Duration of Protection with 2% Pyribenzamine Aerosols.* Three series of 10, 16, and 10 animals were submitted to PBZ aerosol inhalation of 15, 30, and 60 minutes duration respectively. At varying intervals after completion of the PBZ treatment 2 animals of each series received an intracardial injection of 3 mg per kg histamine phosphate (4 MLD).

In the first series of experiments in which the 2% PBZ aerosol was inhaled for 15 minutes, the protection afforded against subsequent injection of 3 mg of histamine phosphate (4 MLD) lasted for at least 1 hour (Table I). Inhalation of PBZ aerosol for 30 minutes gave protection lasting for 3½ hours, and inhalation for 60 minutes did not further increase the protection.

TABLE I.

Duration of Protection in Guinea Pigs Against 4 Lethal Doses of Histamine Phosphate After PBZ Aerosol Inhalation (2%) for 15, 30 and 6 Minutes.

Interval between end of PBZ inhalation and histamine injection	No. of animals protected		
	Duration of aerosol inhalation		
	15 min	30 min	60 min
0	2/2*		
15 min	2/2		
30 ”	2/2	2/2	2/2
45 ”	2/2	2/2	2/2
1 hr	2/2	2/2	1/2
2 ”		2/2	2/2
3 ”		1/2	2/2
3½ ”		2/2	
4 ”		0/4	
Controls, no PBZ		0/6	

* Number of animals protected and surviving over number of animals used.

Anaphylaxis. I. *Influence of the duration of Pyribenzamine Aerosol Inhalation.* Animals actively sensitized to horse serum or passively sensitized with anti-horse rabbit serum could be effectively protected by 2% PBZ aerosol inhalations. The shortest time of inhalation protecting animals against immediate anaphylactic shock was found to be 3 minutes. Animals challenged immediately after this time showed delayed symptoms of shock, but invariably died after 10 to 30 minutes. Complete protection against both shock and anaphylactic death was conferred after an inhalation period of at least 10 minutes.

Since the amounts of histamine or histamine-like substances liberated in the lungs during anaphylactic shock are not known, a direct comparison between the duration of PBZ aerosol inhalation necessary for protection against histamine poisoning and anaphylactic shock is not possible. It is interesting to note, however, that the results in anaphylaxis were somewhat less regular than those obtained in the histamine-shock series, since some animals died in shock even after being submitted to 2% PBZ aerosol inhalation of 30 minutes duration. Contrary to those animals protected against histamine, certain individuals,

TABLE II.
Duration of Protection in Guinea Pigs Against Anaphylactic Shock After PBZ (2%) Aerosol Inhalation for 15 and 30 Minutes.

Interval between end of PBZ inhalation and challenging serum inj.	Number of animals protected		
	Active anaphylaxis after PBZ inhalation of		Passive anaphylaxis after PBZ inhalation of
	15 min	30 min	30 min
15 min	1/2		3/3
30 ”	3/4	2/3	3/3
45 ”	0/3	2/3	2/3
60 ”	2/3	1/2	3/3
2 hr		1/2	2/2
3 ”		2/2	1/1
5 ”			2/2
Controls, no PBZ	0/8		0/3

while protected against immediate anaphylactic shock by adequate treatment, died during the ensuing 24 hours.

II. *Duration of the Protection with 2% Pyribenzamine Aerosol Inhalation.* The duration of the protection to anaphylactic shock conferred to guinea pigs by PBZ aerosol inhalation was tested in the same manner as described in the experiments with histamine. Two groups of 12 sensitized animals each were submitted to an inhalation of 2% PBZ aerosol for 15 and 30 minutes respectively. It was found that inhalation of 15 minutes duration afforded protection up to 1 hour; while inhalation for 30 minutes gave protection for at least 3 hours. Protection was even greater and more consistent in 17 passively sensitized animals, since aerosol inhalation for 30 minutes was still effective after an interval of 5 hours (Table II).

III. *Influence of Aerosols Containing Trasentin or Procaine upon Histamine Intoxication.* PBZ and other antihistaminic substances display, in addition to the antihistaminic activity, powerful local anesthetic and variable antispasmodic activities. The possibility has been suggested that the activity of the antihistaminic substances, especially their effectiveness in anaphylaxis and allergy, are at least partially due to these secondary activities. Although it is known that local anesthetics and antispasmodics, such as atropine, are capable of protecting animals to a limited degree against histamine poisoning and anaphylactic shock, there is no proof that either of these secondary activities associated with antihistaminics contribute to any significant degree to the antihistaminic and anti-anaphylactic activities of PBZ and similar substances. Nevertheless, it seemed interesting to substitute for aerosols of PBZ, aerosols of substances with pronounced antispasmodic and local anesthetic activity and to investigate their influence upon histamine intoxication. We found that neither 2% procaine nor 2% Trasentin aerosol inhalation had any influence upon histamine intoxication using 2 to 3 lethal doses of histamine; convulsions and death occurred at the same intervals as in control animals not submitted to these aerosols.

IV. *Local Action of Pyribenzamine Inhalation upon the Lungs.* As it is known that various antihistaminic substances are irritating when injected subcutaneously, it was therefore necessary to control the effect of PBZ aerosol inhalation upon the cells of the lung system. Guinea pigs were subjected to inhalation of 2% PBZ for up to 2 hours and then observed for several days. The treatment was well tolerated and did not produce any clinical signs of irritation either during treatment, or subsequently. Three other animals, after receiving a 1-hour aerosol treatment, were killed 24 hours later and the lungs histologically examined. They were found to be normal.

Discussion. These experiments indicate that it is possible to protect guinea pigs against 15 lethal doses of histamine, as well as against anaphylactic shock when 2% PBZ is administered in the form of an aerosol. Under the conditions of our experiments, the animals were protected after having inhaled, by calculation, 0.3 to 0.5 mg PBZ, which may have been absorbed, in whole or in part, by the lungs. This dose, as we have found from all our previous experiments, is ineffective when either injected subcutaneously or given orally. These results constitute, in our opinion, further evidence supporting the hypothesis that PBZ specifically counteracts histamine not by central action, but by a purely local influence at the very site of the peripheral histamine action.[2,3,7,8]

The evidence that the point of attack by PBZ is the peripheral receptor may explain why the protection against histamine intoxication or anaphylactic shock, persists for a surprisingly long period of time in spite of the exceedingly small amounts of PBZ which reach the lung tissue during the brief aerosol treatment. Indeed, the protective effect lasts almost as long as that protection which is conferred when PBZ is given subcutaneously or by mouth in considerably higher dosages.

It may be concluded from our results that PBZ is either selectively fixed within the receptor cells of the lungs and does not rapidly enter the blood stream as do most other substances introduced therapeutically into the lungs, or that the minute amounts of antihistaminic substances, by direct contact with the cells susceptible to histamine, render the receptive organs, by some functional mechanisms, refractive for long periods of time to subsequent attacks by histamine.

The introduction of antihistaminic substances into the lungs and the selective protection of this organ against histamine in guinea pigs have enabled us to detect signs of histamine intoxication previously not observed. Under normal conditions, the picture of histamine intoxications in guinea pigs consists almost entirely of the constriction of smooth muscles and its immediate consequences, and before any other signs can develop, the animal dies of asphyxia. On the other hand, smaller doses which do not produce this type of death are apparently too small to produce other symptoms. However, when the animals are protected against death by asphyxia, as in the present experiments, histamine in significantly high dosage can be introduced and unusual symptoms are observed, as described in these experiments; namely, a pronounced narcotic effect which lasts from 30 minutes to 1 hour. At the end of this period the animals slowly recover, as from deep narcosis.

It may be more than a fortuitous occurrence that in certain patients the various antihistaminic substances exert a definite depression and an almost narcotic action. This symptom may be due to the local anesthetic power of these substances. It also could be explained in the light of the foregoing observations; namely, that the competitive action of PBZ with histamine, in some instances, may lead to a duplication of certain histamine effects; while in others, to a suppression of other typical histamine activities.

PBZ aerosol inhalation not only confers a powerful protection against histamine intoxication in guinea pigs, but also protects against anaphylaxis. The doses necessary to prevent anaphylactic shock are approximately the same as those which are active against histamine poisoning, and in both cases the duration of protection is essentially the same. The fact that exceedingly small doses of PBZ introduced directly into the lungs are equally effective in both histamine poisoning and anaphylaxis adds another argument in favor of the theory that anaphylactic death and histamine death in guinea pigs are produced by the same poison, or at least by substances closely related.

Conclusions. 1. PBZ inhaled in the form of an aerosol and in minute amounts, protects guinea pigs against histamine intoxication and anaphylactic shock. This protection is relatively long-lasting. 2. The selective protection which PBZ aerosols confer to the lung tissue permits the observation in guinea pigs of hitherto unrecorded histamine effects. 3. The high activity of PBZ aerosol inhalation and the apparent lack of irritation suggest the possible therapeutic use of PBZ as an aerosol, since it may be predicted that this method of administration produces a lasting therapeutic effect with doses too small to produce undesirable side reactions.

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[8] Yonkman, F. F., Chess, D., Mathieson, D., Hansen, N., *J. Pharm. Exp. Ther.*, 1946, **87**, 256.

15711

In vitro Observations of the Mode of Action of Streptomycin.*

Elias Strauss. (Introduced by S. Edward Sulkin.)

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Despite wide clinical interest in the use of streptomycin, the mode of action of this substance on bacteria is as yet poorly understood. The present report deals with 3 aspects of the action of streptomycin on bacteria *in vitro*: (1) morphological effects of streptomycin on growing cultures of various organisms; (2) the bactericidal action of streptomycin on resting bacteria; and (3) the effect on the rate of growth of bacteria of subinhibitory concentrations of streptomycin.

Effect of Streptomycin on Morphological Appearance of Bacteria. Little attention has been directed toward possible alterations in bacterial morphology as a result of growth in the presence of streptomycin. Welch, Price, and Randall[1] observed huge and bizarre forms of *E. typhosa* in cultures exposed to streptomycin. However, Miller and Bohnhoff[2] found no morphological changes in meningococci or gonococci grown in the presence of streptomycin.

In the present study, a single stock laboratory strain of each of the following organisms was employed: *Aerobacter aerogenes, Escherichia coli, Proteus ammoniae, Pseudomonas aeruginosa, Shigella sonnei, Eberthella typhosa, Salmonella typhimurium, Vibrio comma, Micrococcus tetragenus, Staphylococcus aureus, Bacillus anthracis, Bacillus mycoides, Bacillus subtilis,* and *Bacillus megatherium.* Preliminary tests were performed in heart infusion broth by the serial dilution technic to determine the range of sensitivity of each organism to streptomycin. The degree of bacteriostasis was estimated by the presence or absence of turbidity after 24 hours' incubation. In the final tests, 10^{-2} dilutions of overnight growths of organisms in broth were incubated at 37°C in heart infusion broth in the presence of various amounts of streptomycin, which, on the basis of the preliminary study, were expected to be slightly, moderately, or strongly bacteriostatic. Replicate tubes were prepared for each organism with the several streptomycin concentrations, as well as control tubes. At 0, 3 and 5 to 6 hours' incubation, the tubes were centrifuged for 20 minutes and Gram-stained smears of the bacterial sediment prepared. In each instance, a streptomycin-free culture was smeared and stained on the same slide as the streptomycin-containing culture. The stained smears were examined independently by 2 observers, one of whom was unaware of the purpose or technic of the study.

The effect of streptomycin on the size, shape and staining qualities of bacteria was variable, being marked with some Gram-negative bacilli, slight with Gram-positive cocci, and absent with Gram-positive bacilli. The most extreme alterations in appearance were found with the strain of *Shigella sonnei.* Cells of this organism, when exposed to streptomycin, became elongated (up to 3 or 4 times as long as the control cells), appeared swollen, and stained irregularly. (Fig. 1). These alterations were more pronounced with a slightly bacteriostatic amount of streptomycin (1 γ per ml) than with a higher, more strongly bacteriostatic concentration (5 γ per ml). Cells of changed appearance were somewhat more frequent after 6 hours' incubation than after 3 hours.

Cells of the strain of *S. typhimurium,* when exposed to streptomycin, became swollen and elongated (up to 2 or 3 times the normal length), and assumed coccoid forms. These changes were equally prominent after 3 and 6 hours' incubation. The proportion of altered cells to the total was greater with

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* This investigation was supported by grants from the Lederle Laboratories Division, American Cyanamid Company, and from the United States Public Health Service.

[1] Welch, H., Price, C. W., and Randall, W. A., *J. Am. Pharm. Assn.*, 1946, **35**, 155.

[2] Miller, C. P., and Bohnhoff, M., *J. Am. Med. Assn.*, 1946, **130**, 485.

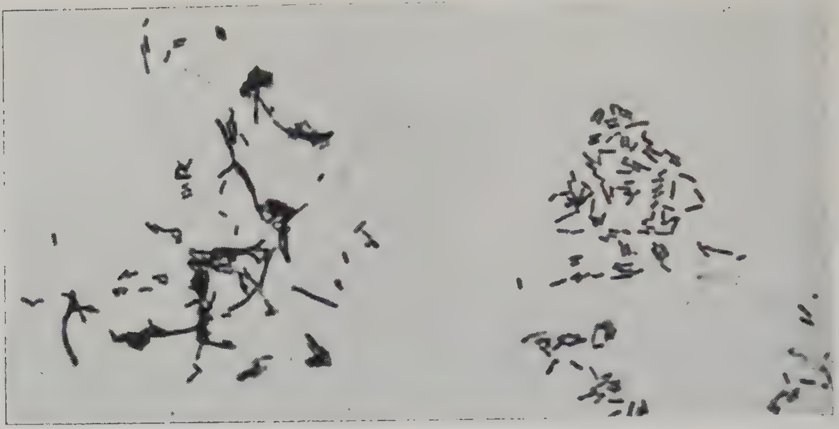

FIG. 1.

Shigella sonnei (× 675), Gram-stained smears after 6 hours' incubation at 37°C in heart infusion broth. Left: In the presence of streptomycin, 1.0 γ per ml. Right: Control, without streptomycin.

slightly bacteriostatic concentrations of streptomycin (3 and 6 γ per ml) than with a strongly bacteriostatic concentration (12 γ per ml).

Definite, but lesser, degrees of alteration in cellular appearance were observed with the strains of *Aerobacter aerogenes* and *Proteus ammoniae.* In both instances incubation with streptomycin for either 3 or 6 hours resulted in a greater proportion of moderately elongated and swollen cells than were observed in control cultures incubated in the same manner without streptomycin. Again the changes were somewhat more frequent in slightly bacteriostatic amounts of streptomycin (1 γ and 3 γ per ml) than in more strongly bacteriostatic concentrations (6 γ and 12 γ per ml).

The other Gram-negative organisms exhibited little or no alteration in appearance. *Vibrio comma* appeared somewhat more pleomorphic in the presence of streptomycin than in its absence. No consistent changes were observed in the appearance of *Escherichia coli, Eberthella typhosa,* and *Pseudomonas aeruginosa.*

A few swollen and deeply-stained cells of *Staphylococcus aureus* and *Micrococcus tetragenus* were found in cultures containing streptomycin but such cells were also seen with nearly the same frequency in control cultures. The Gram-positive bacilli studied exhibited no consistent alterations in shape, size, or staining as a result of incubation with

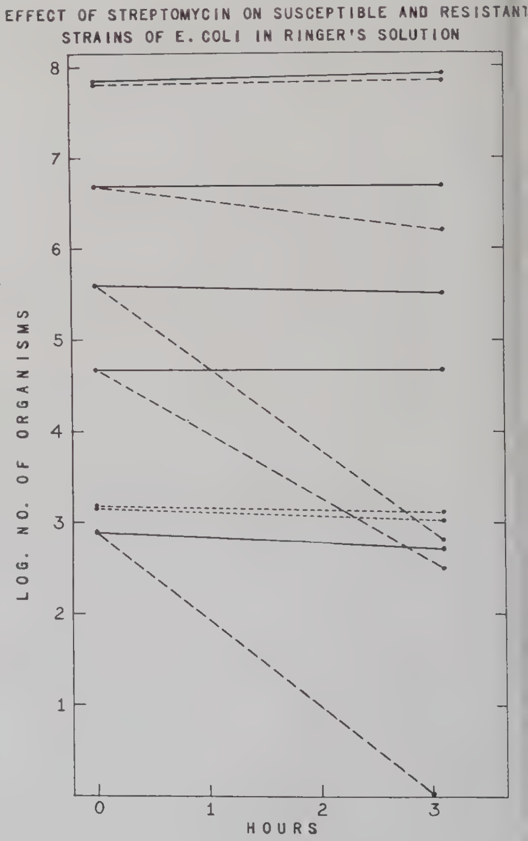

FIG. 2.

Bactericidal action of streptomycin on sensitive and resistant strains of *E. coli* suspended in Ringer's solution at 37°C. Solid lines: Sensitive strain, without streptomycin. Broken lines: Sensitive strain, with streptomycin, 25 γ per ml. Dotted lines: Resistant strain, without streptomycin, and with streptomycin 25 γ per ml.

streptomycin.

Bactericidal Action of Streptomycin on Resting Bacteria. Hamre, Rake, and Donovick[3] reported that streptomycin was bactericidal for *K. pneumoniae* in Ringer's solution. The effect was related to the concentration of streptomycin employed. A greater concentration was required for bactericidal action when the bacterial cells were in the resting state than when they were actively growing. Resting cells surviving 3 or 5 hours' incubation with streptomycin subsequently were not found more resistant than controls. It was suggested, therefore, that the mode of action of streptomycin on washed, resting cells is different from its action on multiplying organisms. Klein and Kimmelman[4] also noted that streptomycin was bactericidal for washed suspensions of *Shigellae* and that the survivors did not show increased resistance. The present studies were undertaken to describe more fully the bactericidal action of streptomycin on resting bacteria and to ascertain whether or not its mode of action under these circumstances differed from its action on multiplying organisms.

A stock laboratory strain of *E. coli* was used. The sedimented organisms from a 6-hour growth in heart-infusion broth were washed twice in Ringer's solution (pH 7.0), and resuspended in Ringer's solution after dilution to the desired bacterial concentrations. Streptomycin in Ringer's solution was added to some of the tubes and the volumes of both control and drug-containing tubes were adjusted. Incubation was carried out in a water bath at 37°C for 3 hours. Explants were made at 0 and 3 hours in a simple synthetic medium[5] to which were added agar, glucose (1%), Bacto tryptose (1%) and sodium thioglycolate (0.05%). Preliminary tests indicated that in this medium concentrations of streptomycin up to 5 γ per ml had no inhibitory effect on the number of developing colonies as compared with controls. In heart-infusion broth assays, the strain was inhibited by 1.6 γ per ml or less of streptomycin.

The results of one set of experiments are shown in Fig. 2. The bactericidal action of streptomycin was related, in general, to the concentration of organisms. Bacterial suspensions of 1000 per ml or less were sterilized in 3 hours by 25 γ per ml of streptomycin; suspensions of 10 million or more cells per ml were not affected by this concentration of streptomycin in the same time interval. Intermediate numbers of organisms were affected proportionately. The activity of 50 γ per ml of streptomycin was greater and that of 10 γ per ml was correspondingly less than the effects indicated with 25 γ per ml. The amount of streptomycin required to sterilize a resting suspension of *E. coli* within 3 hours was approximately 25 times as great as the bactericidal concentration for a similar number of organisms incubated in heart-infusion broth.

An attempt was made to increase the resistance of *E. coli* by repeated exposure of resting organisms to sublethal concentrations of streptomycin. After growth in heart-infusion broth the organisms were washed twice, resuspended in Ringer's solution containing 10 γ per ml of streptomycin, and incubated for 3 hours at 37°C. A loopful of the bacterial suspension was then streaked on an agar plate. After incubation the growth from the agar was again washed and resuspended for 3 hours in Ringer's solution containing 10 γ per ml of streptomycin. This procedure was carried out through 12 passages. A control strain was treated in an identical manner except for the omission of streptomycin. At the completion of the series, both strains were tested for resistance to streptomycin. Neither exhibited any increased resistance to the bactericidal action of streptomycin, as compared with unexposed control strains, when tested in the resting state or when incubated in heart-infusion broth. These results, which are in agreement with the reports of others, suggest a difference in the mode of action of streptomycin on resting and on multiplying organisms.

To further elucidate the mechanism of the mode of action of streptomycin, observations

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[3] Hamre, D., Rake, G., and Donovick, R., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 25.

[4] Klein, M., and Kimmelman, L. J., *J. Bact.*, 1946, **52**, 471.

[5] Sahyun, M., Beard, P., Schultz, E. W., Snow, J., and Cross, E., *J. Infect. Dis.*, 1936, **58**, 28.

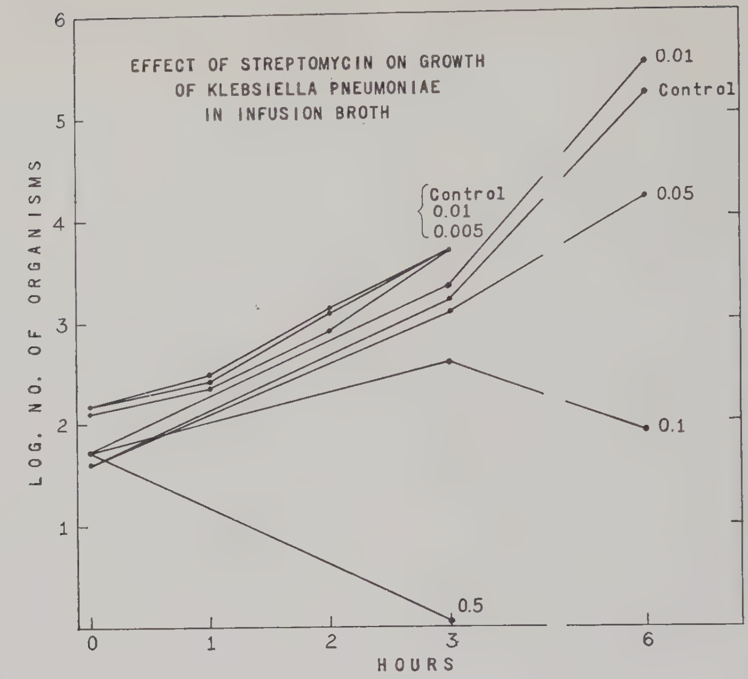

FIG. 3.
Rate of growth of *K. pneumoniae* in heart-infusion broth at 37°C in presence of bactericidal, bacteriostatic, and subinhibitory concentrations of streptomycin. Numbers indicate γ per ml of streptomycin.

were made of the behavior of a strain of bacteria made resistant to streptomycin and subsequently exposed in the resting state to the antibiotic. Accordingly, the stock strain of *E. coli* was made resistant by repeated passage in increasing concentrations of streptomycin in tryptose-phosphate broth. After 12 daily passages, the organisms grew freely in 150 γ per ml of streptomycin. The resistant organisms were then suspended in Ringer's solution and exposed to streptomycin by the procedure already described. A representative result is shown in Fig. 2. This strain was completely resistant to 25 γ per ml of streptomycin—an amount of streptomycin which was bactericidal for a similar number of nonresistant organisms. The mechanism of bacterial resistance to chemotherapeutic agents is presumed to be intimately related to the mechanism of the antibacterial action of such drugs. These results, therefore, suggest that the mode of action of streptomycin on nonmultiplying organisms is essentially similar to its action on multiplying organisms.

Effect of Subinhibitory Concentrations of Streptomycin on Rate of Growth. On the basis of *in vivo* studies in mice, the suggestion was advanced that subinhibitory concentrations of streptomycin might have a stimulatory effect on the rate of growth of *E. typhosa*.[1] This possibility was investigated *in vitro* using a stock strain of *K. pneumoniae*. The organisms were grown overnight in heart-infusion broth, suitably diluted, and inoculated in broth containing various amounts of streptomycin. Explants in tryptose-phosphate agar were made at intervals after incubation at 37°C, and the subsequent growth of colonies counted. The results of 2 experiments are shown in Fig. 3. With this strain of bacteria, 0.5 γ per ml of streptomycin was bactericidal in 3 hours, and 0.1 and 0.05 γ per ml were moderately or slightly bacteriostatic. Lesser concentrations of streptomycin did not appreciably modify the rate of growth of the organisms as compared with the drug-free controls. Colony counts in explants made at hourly intervals during the early phases of growth likewise yielded no evidence of an initial stimulus to the rate of growth from the presence of subinhibitory concentrations of streptomycin.

Comment. The morphological changes in bacteria resulting from streptomycin appear to be less marked than those which occur with exposure to penicillin.[6-9] Like the latter, however, they occur with concentrations of streptomycin which are only slightly bacteriostatic. Alterations in morphology are not regularly associated with bacteriostasis. The growth of all of the organisms studied was inhibited by streptomycin, in concentrations varying between 1 and 50 γ per ml, but only a few strains exhibited abnormalities in size and shape.

Because of its pronounced effect on bacterial morphology, as well as its failure to sterilize nonmultiplying cultures, penicillin is presumed to interfere directly with bacterial fission. In the case of streptomycin it is suggested that the observed morphological changes may be secondary effects resulting from disturbances of intermediary metabolism rather than primary effects on fission. Such an hypothesis is also consistent with the observed bactericidal action of streptomycin on resting, nonmultiplying cells.

The failure of resting cells to develop resistance to streptomycin and the greatly increased concentrations of antibiotic required to effect sterilization in the resting state, as contrasted with the multiplying state, suggest that streptomycin may act on a different locus or by a different mechanism under the 2 conditions of study. However, cells made resistant to streptomycin under conditions of growth are also resistant in the resting state. This observation appears to support the view that the mechanism of action of streptomycin on resting and on multiplying cells is fundamentally the same, the observed differences in behavior being accounted for on the basis of differences in the rate of bacterial metabolism.

Summary. 1. Streptomycin in bacteriostatic concentrations produced morphological changes in some Gram-negative bacilli but not in others. Gram-positive cocci were only slightly affected, and Gram-positive bacilli were not affected. 2. Streptomycin was bactericidal for *E. coli* in the resting state, the effect being related both to the size of the inoculum and the concentration of the antibiotic. 3. Strains of *E. coli* resistant to streptomycin under conditions permitting growth were also resistant in the resting state, but exposure of susceptible strains to streptomycin in the resting state did not result in increased resistance. 4. No stimulation of the rate of growth of *K. pneumoniae* was observed *in vitro* by subinhibitory concentrations of streptomycin.

Grateful acknowledgment is made to Elizabeth Watson for assistance with the morphological studies.

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[6] Gardner, A. D., *Nature*, 1940, **146**, 837.

[7] Miller, C. P., Scott, W. W., and Moeller, V., *J. Am. Med. Assn.*, 1944, **125**, 607.

[8] Thomas, A. R., and Levine, M., *J. Bact.*, 1945, **9**, 623.

[9] Alture-Werber, E., Lipschitz, R., Kashdan, F., and Rosenblatt, P., *J. Bact.*, 1945, **50**, 291.

15712

Similarities in Electron Micrographs of Purified Lansing and SK Poliomyelitis Virus.

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This note is written to correct the impression which was erroneously given[1] that purified SK poliomyelitis virus consists of asymmetric particles of the type described by

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[1] Loring, H. S., Marton, L., and Schwerdt, C. E., Proc. Soc. Exp. Biol. and Med., 1946, **62**, 291.

Gard[2] for a strain of Theiler's mouse virus. While this conclusion was suggested by one of Bourdillon's[3] experiments in which an older dialyzed, purified preparation showed double refraction of flow, the results published by Jungeblut and Bourdillon[4] show a remarkable agreement between purified SK and our purified Lansing preparations. The purified SK samples in contrast to those from unpurified tissue cultures showed the presence of "spherical or elliptic bodies" having a diameter between 25 and 30 $m\mu$. It is noteworthy that particles of the same relative size and shape have been obtained, therefore, from 2 different strains of poliomyelitis virus, both of which were isolated originally from infectious human material. As it might be expected that different human strains would have similar physical properties, the results from the 2 laboratories confirm the fact that human poliomyelitis virus as cultivated in mice and in cotton rats consists of relatively spherical particles of about 25 $m\mu$ diameter.

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[2] Gard, S., *Acta Med. Scand.*, Suppl., 1943, **143**, 173p.

[3] Bourdillon, J., *Arch. Biochem.*, 1943, **3**, 285.

[4] Jungeblut, C. W., and Bourdillon, J., *J. Am. Med. Assn.*, 1943, **123**, 399.

15713

Further Studies of Factor R.*

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Bauernfeind, Schumacher, Hodson, Norris, and Heuser[1] announced the existence of a new factor required for growth and reproduction in the domestic fowl. Schumacher, Heuser, and Norris[2] presented evidence that the factor consisted of 2 components; factor S precipitated from an acid solution by alcohol, and factor R precipitated by alcohol from neutral solution.

While these studies on factors R and S were in progress, evidence of the existence of a factor required for the growth of *L. casei* and *S. faecalis* was presented by various laboratories. This factor is now generally referred to as folic acid. Briggs, Luckey, Elvehjem, and Hart[3] reported that chicks grew normally on a diet containing no more than 8 μg of folic acid per 100 g of diet. Hill, Norris, and Heuser[4] concluded that factor R was not identical with folic acid, and that if folic acid was required by the chick, the amount needed was less than 15 μg per 100 g of diet.

Binkley and associates[5] reported the presence of vitamin B_c (folic acid) conjugate in yeast. This substance was highly active for the chick, but only slightly active for *S.*

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* This work was supported in part by the establishment of grants at Cornell University by the Cerophyl Laboratories, Inc., Kansas City, Mo., The Nutrition Foundation, Inc., New York, N.Y., and the Wyeth Institute of Applied Biochemistry, Philadelphia, Pa.

† Present address, Department of Chemistry, Colorado A. and M. College, Fort Collins, Colo.

[1] Bauernfeind, J. C., Schumacher, A. E., Hodson, A. Z., Norris, L. C., and Heuser, G. F., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 108.

[2] Schumacher, A. E., Heuser, G. F., and Norris, L. C., *J. Biol. Chem.*, 1940, **135**, 313.

[3] Briggs, G. M., Jr., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1945, **158**, 303.

[4] Hill, F. W., Norris, L. C., and Heuser, G. F., *J. Nutrition*, 1944, **28**, 175.

[5] Binkley, S. B., Bird, O. D., Bloom, E. S., Brown, R. A., Calkins, D. G., Campbell, C. J., Emmett, A. D., and Pfiffner, J. J., *Science*, 1944, **100**, 36.

faecalis and *L. casei.* Concentrates of this substance, however, became active for the microorganisms after enzymatic digestion.

In view of the results of Binkley and associates,[5] further work on factor R was undertaken in this laboratory to determine if its activity for chicks could be accounted for in terms of folic acid, as measured by microorganisms after enzymatic conversion of the conjugate to folic acid.

While the study reported in this paper was in progress, Pfiffner and associates[6] reported the isolation of vitamin B_c conjugate in crystalline form from yeast. The vitamin B_c present in the conjugate was found to be available to the chick, and became available to the microorganism after digestion with hog kidney conjugase.

Experimental. The experimental procedure consisted of treatment of factor R with folic acid liberating enzymes, determination by microorganisms of the folic acid in the treated preparations, and comparison with the amount of folic acid present as evidenced by growth and hemoglobin formation in the chick.

Microbiological Work. Folic acid assays were made by a modification of the procedure of Luckey, Briggs, and Elvehjem[7] using *S. faecalis.* The standard of comparison was either synthetic folic acid of Angier and associates[‡,8] or a folic acid concentrate supplied by Dr. R. J. Williams. One μg of synthetic folic acid was found to be equivalent to 11.5 μg of Williams' folic acid. Values, obtained when Williams' folic acid was used as the standard, were converted to synthetic folic acid by means of this factor.

The amount of free folic acid in factor R was determined following either incubation of the sample with takadiastase and papain or steaming in phosphate buffer at pH 7 for 15 minutes. Both methods of extraction gave comparable values. The results showed that there was not sufficient folic acid present as such to account for the growth response of the chick to factor R. Therefore, other enzymatic digestions were undertaken to determine the presence of potential or conjugated folic acid that was microbiologically inactive, but available to the chick.

In preliminary work the most effective procedure found was one involving a 24-hour predigestion at 37°C with malt diastase, followed by a 24-hour incubation at 37°C with fresh rat liver suspension. The entire procedure was carried out in citrate-phosphate buffer at pH 4.5. This treatment was applied to factor R and to a Superfiltrol eluate of factor R, and consistently gave an approximately 10-fold increase in folic acid content. Chick liver, in general, was less effective than rat liver. Increases of this order of magnitude still, however, did not adequately account for all the biological activity of factor R. Hence, resort was made to the use of dried kidney suspension, as described by Bird and associates.[9] This procedure resulted in an approximately 15-fold increase in folic acid content.

Chick Experiments. Two experiments were conducted with day-old White Leghorn chicks. Not less than 15 chicks were started in each lot. They were housed in batteries of individually heated, well lighted pens with raised wire mesh bottoms.

The basal diet used was purified diet 653 described by Hill, Norris and Heuser.[4] Factor S, prepared according to the method of Schumacher, Heuser, and Norris,[2] was supplied at a level equivalent to 5% of dried brewers' yeast, and 100 μg of 2-methyl-3-

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[6] Pfiffner, J. J., Calkins, D. G., O'Dell, B. L., Bloom, E. S., Brown, R. A., Campbell, C. J., and Bird, O. D., *Science*, 1945, **102**, 228.

[7] Luckey, T. D., Briggs, G. M., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **152**, 157.

[8] Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., SubbaRow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr., *Science*, 1945, **102**, 227.

‡ We are indebted to Lederle Laboratories, Pearl River, N.Y., for synthetic folic acid, to Dr. R. J. Williams, University of Texas, Austin, Tex., for folic acid concentrate, to Merck & Co., Inc., Rahway, N.J., for β-pyracin, and to Anheuser-Busch, Inc., St. Louis, Mo., for strain S dried brewers' yeast.

[9] Bird, O. D., Bressler, B., Brown, R. A., Campbell, C. J., and Emmett, A. D., *J. Biol. Chem.*, 1945, **159**, 631.

hydroxy-4-carboxy-5-hydroxymethylpyridine (β-pyracin) were added per 100 g of diet.

The chicks were weighed individually at weekly intervals. Hemoglobin determinations were made at the 3rd, 4th, and 6th weeks in Experiment 1 by the colorimetric method of Sahli, using 0.1 N HCl to develop the acid hematin color, and in Experiment 2 by the oxyhemoglobin method developed in this laboratory by Robertson and Fiala,[10] using 0.1% Na_2CO_3, and measuring the color produced with a Coleman spectrophotometer.

Experiment 1. The *L. casei* factor of Hutchings and associates,[11] was used as a standard. The sample used was approximately 80% pure and was added at levels of 50, 100 and 150 μg per 100 g of diet. Factor R was included at levels equivalent to 5, 10, and 15% of original yeast. The results of the experiment indicated that 50 to 100 μg of *L. casei* factor were needed for optimum growth response in the chick. Using the same experimental conditions, Robertson, Daniel, Farmer, Norris, and Heuser[12] have reported that 45 to 55 μg of synthetic folic acid per 100 g of diet were required for optimum growth. Normal growth was obtained with factor R preparations that supplied approximately 25 μg of folic acid per 100 g of diet, as determined by *S. faecalis* assay after rat liver incubation.

Experiment 2. When synthetic folic acid became available, an experiment was conducted using 10, 20, 30, and 40 μg of folic acid per 100 g of diet. Factor R was included at levels equivalent to 3, 6, and 12% of original yeast. One group of chicks was supplied the various supplements alone, and a second group the supplements plus 2% succinylsulfathiazole. When the sulfonamide was added, *p*-aminobenzoic acid was omitted from the diet. The results of Experiment 2 are presented in Table I.

The amount of folic acid found in factor R by this chick experiment was approximately 3 μg per g of original yeast. This was obtained by comparing both the growth and hemoglobin responses at the various levels of factor R to the corresponding responses caused by synthetic folic acid. Comparing the biological and microbiological responses to factor R demonstrated that the microörganism was accounting for all of the chick activity after the sample had been incubated with hog kidney, but not when incubated with chick or rat liver.

No significant differences in the chick responses to factor R between the nonsulfonamide and sulfonamide diets were obtained. This indicated that potential folic acid in factor R was not liberated by intestinal bacteria and that under the conditions of the experiment no folic acid was synthesized by the intestinal flora. It appeared, therefore, that either the chick is able to use the folic acid of factor R directly, or metabolic processes make it available.

Discussion. Several explanations for the differences in microbiological results obtained by incubating factor R with chick or rat liver and hog kidney are possible. More than one conjugate of folic acid may be present in factor R. If this is the case, the hog kidney enzyme(s) is able to break down both conjugates, whereas rat and chick liver enzymes can split only one.

The presence of more than one conjugate of folic acid in factor R is supported by the recent findings of Ratner, Blanchard, and Green.[13] These investigators isolated a peptide of *p*-aminobenzoic acid from yeast having 10 or 11 glutamic acid residues. In one of the steps in the isolation of this peptide trichloracetic acid was used. This treatment may have split the peptide from the pterin nucleus of the folic acid conjugate, since Angier and associates[14] brought about the

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[10] Robertson, E. I., and Fiala, G. F., unpublished data, Cornell University, Ithaca, New York.

[11] Hutchings, B. L., Stokstad, E. L. R., Bohonos, N., and Slobodkin, N. H., *Science*, 1944, **99**, 371.

[12] Robertson, E. I., Daniel, L. J., Farmer, F. A., Norris, L. C., and Heuser, G. F., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 97.

[13] Ratner, S., Blanchard, M., and Green, D. E., *J. Biol. Chem.*, 1946, **164**, 691.

[14] Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., SubbaRow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey E. H., Seeger, D. R., Sickels, J. P., and Smith J. M., Jr., *Science*, 1946, **103**, 667.

TABLE I.

Effect of Factor R on Growth and Hemoglobin Formation in the Chick.

Supplements	Weight		Hemoglobin		Folic acid added by supplement			
	4 wk	6 wk	4 wk	6 wk	Unincubated	Hog kidney	Rat liver	Chick liver
	Without succinylsulfathiazole.							
None*	153 (11)†	202 (5)	3.9	2.4	—	—	—	—
10 μg folic acid‡	225 (15)	345 (12)	5.1	6.9	10	—	—	—
20 ” ” ”	249 (15)	412 (15)	8.0	9.5	20	—	—	—
30 ” ” ”	288 (15)	476 (15)	8.3	9.2	30	—	—	—
40 ” ” ”	274 (14)	461 (14)	8.7	9.3	40	—	—	—
3% factor R	174 (12)	266 (9)	3.7	5.1	0.6	9.9	7.5	6.0
6” ” ”	241 (12)	445 (11)	7.8	9.8	1.2	19.8	15.0	12.0
12” ” ”	298 (12)	492 (12)	8.4	9.4	2.4	39.6	30.0	24.0
	2% succinylsulfathiazole added.							
None	153 (8)	173 (2)	3.2	1.6	—	—	—	—
10 μg folic acid	206 (12)	310 (11)	4.8	6.6	10	—	—	—
20 ” ” ”	248 (14)	431 (14)	7.3	9.1	20	—	—	—
30 ” ” ”	282 (14)	469 (14)	8.2	9.5	30	—	—	—
40 ” ” ”	281 (14)	470 (14)	8.5	9.5	40	—	—	—
3% factor R	186 (12)	230 (8)	4.4	3.5	0.6	9.9	7.5	6.0
6” ” ”	239 (15)	413 (11)	6.2	9.1	1.2	19.8	15.0	12.0
12” ” ”	301 (15)	492 (15)	8.0	9.2	2.4	39.6	30.0	24.0

* Basal diet contained 15 μg of folic acid per 100 g as determined by *S. faecalis* assay following chick liver incubation.

† Numbers in parentheses indicate number of surviving chicks of the original 15.

‡ Synthetic folic acid.

cleavage of the fermentation *L. casei* factor giving a pteridine fraction and an aromatic amine by using sulfurous acid, which is a milder treatment than that with trichloracetic acid.

The vitamin B_c conjugate of Pfiffner and associates,[15] which is also obtained from yeast, has been shown to contain 7 molecules of glutamic acid. This evidence indicates that there may be at least 2 different conjugates in yeast. If there are 2 conjugates of folic acid present in factor R, it may be that there are 2 enzymes needed to split them, both of which are present in hog kidney and only one in rat and chick liver.

All conditions necessary for the optimum efficiency of the rat and chick liver enzyme systems may not have been met, so that complete liberation of folic acid from the conjugate was not obtained. Every effort was made to determine the optimum conditions for the enzyme systems involved. The proper concentrations of the enzymes and substrate were determined, as well as the optimum pH and temperature for the incubation.

Hog kidney may contain an enzyme system that can synthesize folic acid from simple precursors, whereas chick and rat liver may be devoid of such an enzyme. Many bacteria, yeasts, and molds are known to possess enzyme systems that are able to take the pyrimidine and thiazole fractions of thiamine and combine them into the vitamin.

Summary. The growth and hemoglobin responses obtained in chicks receiving a purified diet supplemented with factor R were found not to be due to the preformed folic acid present in factor R.

By means of chick liver, rat liver, and hog kidney incubation procedures the folic acid content of factor R was greatly increased. This indicated the presence of potential folic acid which did not stimulate the growth of *S. faecalis,* but which was available to the chick. The potential folic acid liberated by hog kidney enzymes was found to account for all of the responses obtained in the chick, whereas chick and rat liver enzymes were not as effective in liberating folic acid.

The most probable explanation for the discrepancy in the folic acid content of factor R obtained following hog kidney and rat or chick liver incubations is that factor R is a mixture of conjugates of folic acid rather than one conjugate.

The response to factor R was not affected by the presence of succinylsulfathiazole in the diet. Conjugated folic acid appeared, therefore, to be available as such to the chick, or was made available by metabolic processes.

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[15] Pfiffner, J. J., Calkins, D. G., Bloom, E. S., and O'Dell, B. L., *J. Am. Chem. Soc.*, 1946, **68**, 1392.

15714

Nerve Conduction After Inactivation of Choline Esterase.*

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Gilman reported in February, 1946[1] that di-isopropyl fluorophosphate (DFP) could block conduction in a stretch of frog nerve dipped in it; but that conduction returned on lifting out the nerve. Despite this "reversibility" of the block, the choline esterase (Ch.E.) was left inactivated. Objections were raised to the conditions of these experiments (atypical action potentials, manner of obtaining reversal, low frequency of stimula-

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* The present investigation was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[1] *The Physics and Chemistry of Nerve Conduction,* New York Academy of Sciences Symposium Monograph, in press.

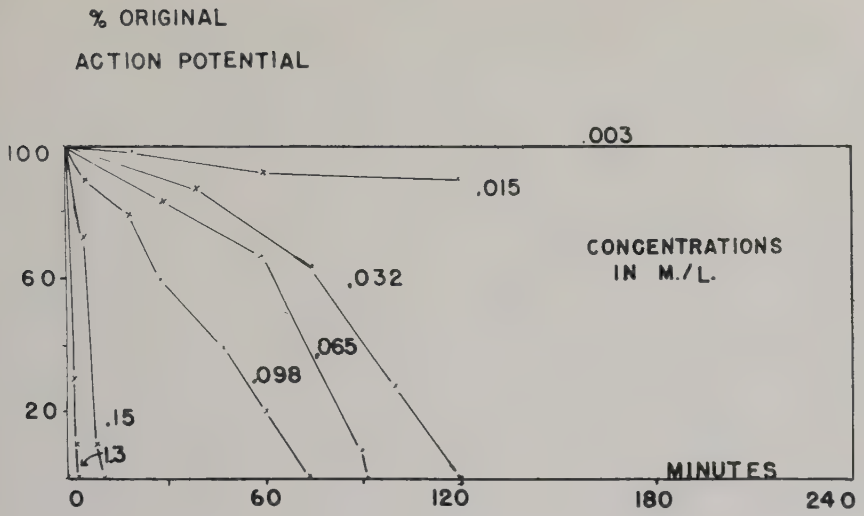

Fig. 1.

tion, small CO_2 values in the manometric determination of Ch.E., inactivation of Ch.E. at the time of grinding of the nerve, etc.).

A few weeks later, at the Federation symposium, succeeding papers by Crescitelli, Gilman, *et al.*[2,3] and by Rothberg, Nachmansohn, *et al.*[4,5] were the basis for an extensive further discussion. The former reaffirmed their findings, with added details; the latter reported that DFP depressed action and Ch.E. in parallel, and that both recovered in parallel on removing the drug, in squid nerve. The attention of other workers was invited to this experiment which, with other evidence (Gerard[1]), seemed potentially conclusive as to the role of A.Ch. in nerve conduction.

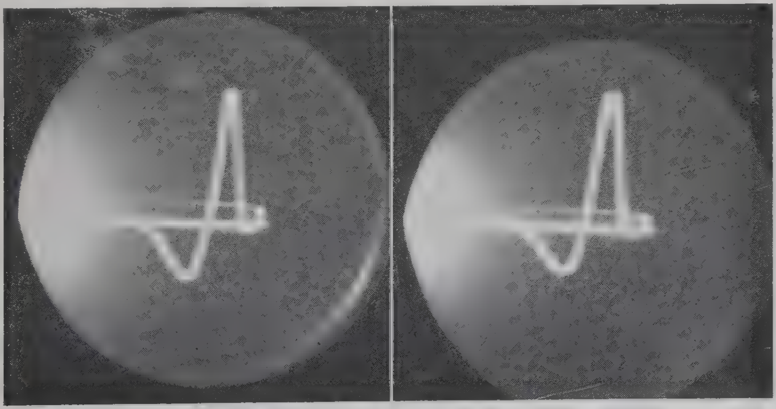

Fig. 2.
Left trace initial; right trace after 5 hours in 3 mM DFP. Read R to L. Conduction distance to first electrode 10 mm; to second one 30 mm. No crush, second electrode near cut end.

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[2] Crescitelli, F., Koelle, G. B., and Gilman, A. F., *Fed. Proc.*, 1946, Pt. II, **5**, 172.

[3] Crescitelli, F., Koelle, G. B., and Gilman, A. F., *J. Neurophysiology*, 1946, **9**, 241.

[4] Rothberg, M. A., and Nachmansohn, D., *Fed. Proc.*, 1946, Pt. II, **5**, 199.

[5] Bullock, T. H., Grundfest, H., Nachmansohn, D., Rothberg, M. A., and Sterling, K., *J. Neurophysiology*, 1946, **5**, 253.

Method. We have modified the procedures as follows. Both sciatics of a frog were immersed in peanut oil, containing the desired concentration of DFP. The nerves rested on stimulating and lead-off electrodes and were tested in place. When desired, to test reversibility, the oil was well washed out of the chamber and fresh oil substituted. Normally, after 3 hours one nerve was removed from the DFP solution for Ch.E. assay, the other left in and its action potentials followed at intervals until the conclusion of the assay. Maximal stimuli (tested repeatedly during an experiment) were used at 20/sec. during the test.

To obviate any possible objection to the CO_2 method, Ch.E. was determined by a direct measure of the A.Ch. at appropriate times after mixing an A.Ch. solution with the homogenized nerve extract. Controls showed that peanut oil traces were without influence on rate of hydrolysis. A.Ch. was assayed on the frog's rectus with the usual precautions. Typical conditions were: 20 to 50 mg of nerve homogenized in ½ cc of Ringer, added to 4.5 cc of 4.5×10^{-4} M A.Ch. in Ringer, assayed after ½, 1, 2 and 3 hours at room temperature, approximately 20°C. At each time interval aliquots of the initial A.Ch. solution and of each of the nerve-A.Ch. suspensions were assayed for A.Ch. activity.[†] The initial solution as assayed was diluted to 0.5 γ A.Ch. Br. in 4 cc Ringer.

Results. In a preliminary series, nerves were followed in various concentrations of DFP until action potentials were abolished or no further change was observed in several hours. Fig. 1 shows a set of results. At DFP concentrations above 15 mM, the action potential progressively falls, during hours or minutes depending on the strength of the solution, to zero. Such a depressed or inactive nerve has never shown a sign of recovery when washed with fresh solvent. (On transfer from oil to Ringer return of potential occurred one time). At DFP concentrations below 15 mM, little depression of the action potential has been observed over a period of 2 hours; at 3 mM, none in over 6 hours.

In the critical experiments, DFP at concentrations of only 3 mM were used. Fig. 2 shows the practically unchanged action potential in a nerve left exposed to 3 mM for 5 hours while its fellow was being assayed for Ch.E. The latter, after 3 hours in the DFP, exhibited zero Ch.E. activity, as shown in Table I. That the enzyme was not inactivated only during homogenization, by DFP previously unable to penetrate, is shown by an experiment in which a DFP nerve and a control nerve were ground together (Table II). Ch.E. activity was more rather than less than the average of poisoned and unpoisoned.

TABLE I.
A.Ch. (as % of original amount) Present After 3 hr Incubation with Ground Nerve.

Exp.	Normal	DFP-treated
1	0	90
2	0	100
3	0	100
4	0	100
5	4	100
6	14	100
7	31	86

TABLE II.

Material tested (after 3 hr incubation)	Muscle response* (arbitrary units) Exp. A	Exp. B
Substrate alone	55	50
Substrate plus DFP-treated nerve	57	55
Substrate plus normal nerve	0	0
Substrate plus DFP and normal nerves	8	12

* Response is proportional to A.Ch. present.

Conclusions. Frog nerve, exposed to DFP in a concentration and for a time sufficient fully to inactivate its choline esterase, can still conduct impulses with entirely normal action potentials. Ch.E. is not essential to nerve conduction.

[†] Comparison of known and unknown solutions at each test is important, especially with DFP present. This substance, added with boiled or unboiled nerve but not by itself, potentiates the muscle response to known A.Ch. With 45 mM DFP, the muscle response may be doubled; at 6 mM the potentiation is around 20%; at 3 mM no potentiation was observed. The basis of this action has not been adequately studied, but no further formation of A.Ch. is involved.

15715 P

Platelet Fragility in Man.

Lewis R. Day. (Introduced by Henry N. Harkins.)

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For many years it has been known that a certain strain of swine demonstrates an abnormality of blood coagulation similar to that found in human hemophiliacs. The genetics of this abnormality and its mechanism have been extensively studied.[1-3]

Working with this strain of swine, Muhrer, Bogart and Hogan[4] have developed a method of testing platelet fragility. It is the purpose of the present study to determine (1) whether the method is applicable to the determination of platelet fragility in man, and (2) whether human platelets react similarly to those of swine.

The method resembles that used for testing erythrocyte fragility. Platelet-rich plasma is obtained by moderate centrifugation of whole blood, drawn with special precautions to minimize trauma to the formed elements. The blood is drawn through a petrolatum-lined No. 20 McCrae needle directly into cold parafinned centrifuge tubes containing sodium citrate. This plasma is added to various hypotonic saline solutions, recalcified, and the time of coagulation measured. It is assumed that the mechanism of coagulation is the classical 2-stage process postulated by most workers in the field, and that coagulation time is quantitatively related to the speed of thrombin formation, which is in turn directly dependent upon the amount of thromboplastin released by platelets. One assumes further that if platelet-rich plasma exposed to hypotonic solutions clots faster than normal plasma, this is due to a more complete lysis of platelets. Conversely, if 2 different specimens, after similar exposure to saline solutions of the same tonicity, have different clotting times, there is presumably a difference in platelet fragility.

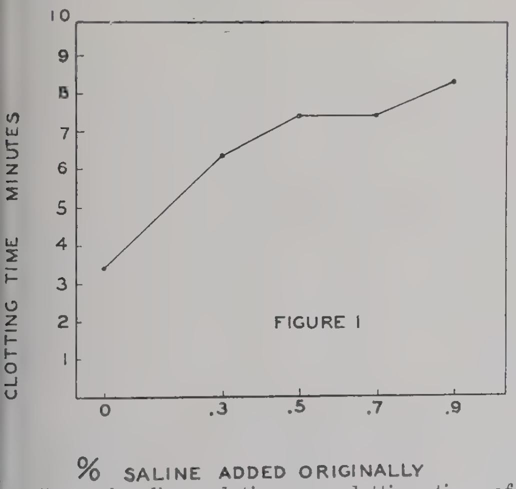

FIGURE 1

Effect of saline solutions on clotting time of normal platelet-rich plasma; reaction taking place in 0.9% saline.

Critique of Method. (1) To determine whether the effect of the hypotonic solutions was on some phase of the clotting mechanism other than the platelets, the specimens, after exposure to the various saline solutions, were all brought up to 0.9% NaCl, prior to recalcification. Under such circumstances, clotting occurred in a normal saline solution. As is shown in Fig. 1, the specimens exposed to hypotonic solutions clotted faster, even though clotting took place in a normal saline solution; and the more hypotonic the solution, the faster was the coagulation on eventual recalcification. The longer the time of exposure to the hypotonic solution, prior to recalcification, the more coagulation was accelerated. (Fig. 2). This suggests that, as in the case of erythrocytes, platelets do not disintegrate all at once, but are slowly lysed over a period of about 15 minutes.

Similar results were obtained with highly centrifuged specimens (Fig. 2). Although smears of these plasmas showed no cells, the

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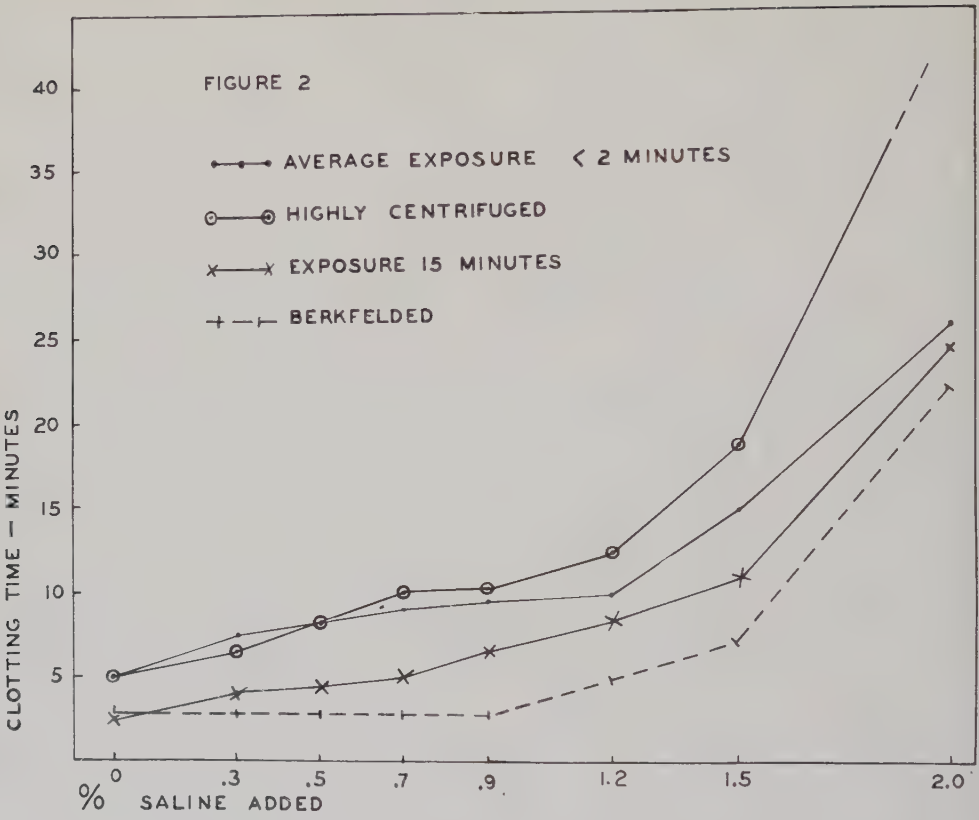
[1] Hogan, A. G., Muhrer, M. E., and Bogart, R., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 217.

[2] Bogart, R., and Muhrer, M. E., *J. Hered.*, 1942, **33**, 59.

[3] Muhrer, M. E., Hogan, A. G., and Bogart, R., *J. Physiol.*, 1943, **136**, 355.

[4] Muhrer, M. E., Bogart, R., and Hogan, A. G., *Am. J. Physiol.*, 1944, **141**, 449.



Effect of saline solutions on clotting time of normal platelet-rich, platelet-poor, and platelet-free plasma and the effect of varying the exposure time; reactions taking place in solutions of varying tonicity.

results were essentially the same. However, it must be assumed that they contained platelets, since when specimens were passed through a Berkefeld N filter, exposure to hypotonic solution no longer affected clotting time significantly (Fig. 2).

These data suggest that the method as described by Muhrer does test the resistance of the platelets to hypotonic saline solution.

Platelet Fragility in Man. (2) The results with several normal human specimens are shown in Fig. 3. These were taken from normal healthy medical students and from white male patients, all of whom had apparently normal clotting mechanisms.

Specimens were also drawn from a known hemophiliac who is 40 years old and has been treated at the Johns Hopkins Hospital since the age of 5. These results are shown in Fig. 3. The curves are similar to those obtained in hemophilic swine, the difference being that in the latter the clotting time was reduced to normal levels by exposure to distilled water, while in man the clotting time was shortened, but not to normal levels.

Discussion. Extensive studies of the clotting mechanism in normal humans and hemophiliacs to determine the fundamental abnormality have evolved several theories.[5-8] The present study on the resistance of normal and hemophilic platelets is offered as additional but not conclusive evidence that the difference between hemophilic and normal blood clotting lies in the platelets and not the plasma. Further study is necessary,

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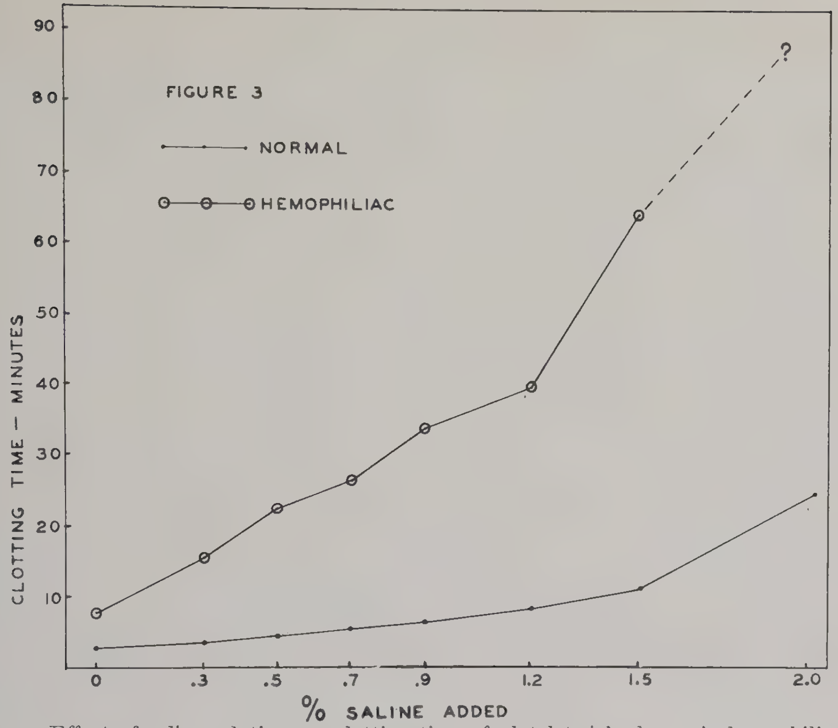
[5] Minot, G. R., and Lee, Roger I., *Arch. Int. Med.*, 1916, **18**, 474.

[6] Howell, W. H., and Cekada, E. B., *Am. J. Physiol.*, 1926, **78**, 500.

[7] Patek, A. J., and Stetson, R. P., *J. Clin. Invest.*, 1936, **15**, 531.

[8] Kark, R., *Clinics*, 1943, **2**, 15.



Effect of saline solutions on clotting time of platelet-rich plasma in hemophilia as compared with normal; reactions taking place in solutions of varying tonicity.

owever, for it is entirely possible that the ncreased lysis of platelets by supplying an xcess of thromboplastin serves to counteract ome other deficiency or excess in hemophilia.

As suggested by Muhrer, one of the chief rawbacks in the testing of substances active n reducing clotting time in hemophilia is the ack of suitable experimental animals. These indings with human blood are sufficiently close to those obtained with the blood of hemophilic swine to suggest the possibility that study of the latter may throw light on the human disease.

I am indebted to Dr. Philip Wagley of the Johns Hopkins Hospital and to Dr. Harry Eagle of the United States Public Health Service for technical aid and advice in carrying out this study.

Present address: Presbyterian Hospital, Chicago.

15716 P

Effect of 2-Methyl-1,4-Naphthoquinone on Glycolysis of *Schistosoma mansoni*.*

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Earlier observations indicated that oxidaive metabolism is not essential for the survival of *Schistosoma mansoni*.[1] Since schistosomes have a high rate of both anaerobic

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* The work described in this report was done nder a contract between the Office of the Surgeon eneral, U. S. Army and Western Reserve University.

[1] Peters, L., Welch, A. D., and Bueding, E., unpublished data.

TABLE I.
Effect of Quinones on Oxygen Uptake and Glycolysis of Schistosomes.

Compound	Molar concn.	Medium	O_2 uptake		Glucose removal		Lactic acid production	
			µl*	% change	µg*	% change	µg*	% change
—	—	Salt	1.21		33.5		30.4	
2-Methyl-1,4-naphthoquinone	$1x10^{-4}$	,,	0.59	—51	0	—100	7.2	—76
,, ,,	$2x10^{-5}$	,,	1.25	+ 3	21.6	— 36	23.2	—24
1,4-Naphthoquinone	$2x10^{-5}$	,,	0.94	—21	13.6	— 58	18.1	—40
1,2- ,,	$2x10^{-4}$	,,	1.15	— 5	28.9	— 13	28.0	— 7
1,4 Quinone	$2x10^{-5}$	,,	1.26	+ 4	24.8	— 26	27.1	—11
,,	$2x10^{-4}$	,,	0.94	—22	7.0	— 79	16.1	—48
1,4-Hydroquinone	$2x10^{-5}$	,,	1.18	— 6	28.2	— 16	28.3	— 7
,,	$2x10^{-4}$	,,	0.87	—28	16.7	— 50	20.9	—32
3-Methoxy-2-methyl-1,4-naphthoquinone	$1x10^{-3}$	,,	0.51	—58	16.7	— 50	13.6	—55
3-Hydroxy-1,4-naphthoquinone†	$1x10^{-3}$	,,	0.58	—52	25.3	— 25	22.3	—27
3-Hydroxy-2-piperidino-methyl-1,4-naphthoquinone†	$1x10^{-3}$	,,	1.19	— 2	29.4	— 12	27.4	—10
3-Hydroxy-2-amyl-amino-methyl-1,4-naphthoquinone†	$1x10^{-3}$	,,	0.80	—34	21.9	— 35	22.3	—27
—		Buffered serum	1.40		37.6		44.6	
2-Methyl-1,4-naphthoquinone	$5x10^{-4}$	,, ,,	1.06	—24	4.9	— 87	13.7	—69
,, ,,	$1x10^{-4}$	,, ,,	1.42	+ 2	23.6	— 37	31.3	—30
1,4-Naphthoquinone	$1x10^{-4}$	,, ,,	1.18	—16	28.3	— 25	36.5	—18
Quinone	$2x10^{-3}$	,, ,,	—	—	12.0	— 68	20.2	—55
Hydroquinone	$2x10^{-3}$	,, ,,	—	—	21.4	— 46	34.9	—22

* Per hour and mg wet wt.

† Kindly supplied by Abbott Laboratories, Chicago.

and aerobic glycolysis, studies have been made of the glycolytic processes and of their inhibition by compounds of low toxicity for mammalian species.

Paired adult schistosomes were removed from the mesenteric or portal veins, or from the larger hepatic vessels of mice infested with *S. mansoni*. The organisms were placed in a glucose-containing salt medium (0.137 *M* NaCl, 0.0027 *M* KCl, 0.0003 *M* $CaCl_2$, 0.001 *M* $MgCl_2$, 0.066 *M* phosphate; pH, 7.4) or in a mixture of 40 volumes of human serum (previously degassed and adjusted to pH 7.4) and 3 volumes of 0.5 *M* phosphate buffer (pH 7.4). A high phosphate concentration is essential for the optimal respiration and glycolysis of schistosomes *in vitro*. Incubation was then carried out in small vessels (vol. 4 to 5 ml) in 0.8 ml of medium at 38.6°C in an atmosphere of air; the consumption of oxygen was measured manometrically. The concentrations of glucose[2] and of lactic acid[3] in the medium were determined before and after the incubation period.

The rate of aerobic glycolysis of schistosomes was inhibited by 2-methyl-1,4-naphthoquinone (representative results are recorded in Table I). Oxygen uptake was inhibited to a much smaller extent than was the removal of glucose or the formation of lactic acid. The concentration of 2-methyl-1,4-naphthoquinone required to produce a 50% inhibition of glycolysis was about 5 times greater in human serum than in a protein-free salt medium. If schistosomes were incubated for 30-60 minutes at 38°C in the serum-medium, containing the naphthoquinone in a concentration of 1×10^{-4} *M*, and were then transferred into fresh medium without the quinone, inhibition of glycolysis was maintained for a period of at least 4 hours. As recorded in Table I, a related compound, 1,4-naphthoquinone, was somewhat more effective than the 2-methyl derivative in inhibiting glycolysis of schistosomes in a salt medium, but it was less effective in serum. 1,2-Naphthoquinone was a much weaker inhibitor of glycolysis of schistosomes than was 1,4-naphthoquinone or 2-methyl-1,4-naphthoquinone. Furthermore, 3-methoxy-2-methyl-1,4-naphthoquinone† and substituted 3-OH-naphthoquinones were much less potent inhibitors of glycolysis of *S. mansoni* than was 1,4-naphthoquinone or 2-methyl-1,4-naphthoquinone. It appears, therefore, that introduction of a hydroxy- or a methoxy-group in position-3 decreases markedly the activity of a naphthoquinone against schistosomes *in vitro*.

When mice infected with schistosomes were fed a diet containing 1 to 1.5% 2-methyl-1,4-naphthoquinone for one week and, during this period, were injected intraperitoneally with subcurative doses of sodium antimony III-biscatechol-2,4 disulfonate ("Fuadin") (7.5 mg/kg every 8 hours for 5 days), the schistosomes removed from the livers of these mice showed a markedly decreased rate of glycolysis, as indicated by 60 to 90% reduction of glucose removal and of lactic acid formation. Furthermore, the combined administration of the antimonial compound and the quinone resulted in the complete disappearance of the schistosomes from the mesenteric veins and in many cases from the portal vein of the host. The administration of 2-methyl-1,4-naphthoquinone alone, or of "Fuadin" alone, resulted in a much smaller decrease in the glycolytic activity (10-20%) of the parasites and did not lead to the exodus of the worms from the mesenteric veins.

Conclusions. 2-Methyl-1,4-naphthoquinone markedly inhibits aerobic glycolysis of *Schistosoma mansoni in vitro*. Since glycolysis, rather than oxidative metabolism, appears to be essential for the survival of these organisms, and since the toxicity of 2-methyl-1,4-naphthoquinone for mammalian species is low, the effect of this compound in experimental schistosomiasis has been studied. Results in mice suggest that the compound may act synergistically with subcurative doses of antimonials ("Fuadin").

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[2] Somogyi, M., *J. Biol. Chem.*, 1945, **160**, 61, 69.

[3] Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, 1941, **138**, 535.

† Kindly supplied by Dr. C. Colwell of The Velsicol Corporation, Chicago.

15717

A Practical Method for Routine Blood Cultures in Brucellosis.

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Various methods have been used in our laboratory for the isolation of Brucella organisms from the blood of patients. Only a minor proportion of the cultures are positive since we are dealing with unselected cases which include patients with other diseases; and because on the brucellosis cases serial blood cultures are performed during the course of the disease. The time-consuming bacteriological procedure and the waste of material entailed in such a large percentage of negative cultures made it advisable to look for other dependable methods for the detection of positive blood cultures avoiding troublesome secondary bacteriological transplants. The method as devised also minimizes the handling of possibly infective material, since such handling has proved dangerous to the technician who sooner or later becomes infected with the melitensis strain. Various procedures were investigated and the one described below has given the most satisfactory results.

The medium used is prepared with bactotryptose. The formula follows:

Bactotryptose	2.0 g
Sodium chloride	0.5 "
Sodium citrate	0.5 "
Agar	3.0 "
Distilled water	100 ml

Fifteen ml of the medium are placed in 100 ml flat-sided, rectangular bottles. The mouth of the bottle (about 1¼ cm in diameter) is covered with thick paper and sterilized at 15 lb for 15 minutes. The bottles are placed on their sides so that the agar sets on one of the narrow sidewalls forming an even, transparent layer. The paper is then lifted, using aseptic technic, and 10 cc of bactotryptose broth are added. The flask is then closed with sterile rubber stoppers and the bottle again covered with the same paper cap. The broth contains 2% bactotryptose and 2% sodium citrate and is sterilized in separate containers at 15 lb for 15 minutes. The air within the bottles can be replaced by suitable mixtures of CO_2 and air by any mechanical device which avoids contamination of the double medium.

Before using this double medium, tests for sterility are made at 37°C for 3 or 4 days. The agar surface is wet by the broth at 24-hour intervals.

The double medium may be inoculated with 10 cc of blood, the patient usually being bled with a 20 cc autoclave-sterilized syringe. The needle is inserted through the rubber stopper after the careful removal of the paper cap. The mixture of blood and broth is spread over the agar layer for a moment and the bottle incubated in a vertical position at 36°C. Every other day the broth is allowed to flow over the agar layer and the bottle replaced in the incubator, again in a vertical position.

Results. When the agar layer shows colonies after 24-48 hours, it is likely that the culture has been contaminated. When the colonies appear 24 or 48 hours after the second inoculation, the cultivated organism has usually been found to be a Salmonella, less frequently Staphylococcus or Streptococcus, but may be Brucella. Positive cultures of Brucella are detected more frequently after the third agar inoculation, that is on the sixth day of cultivation. After 20 days the negative cultures may safely be discarded. The number of colonies and time of their appearance on the agar layer vary from one case to another and serve as a good indication of the intensity of the bacteriemia. Serial cultures performed twice a month have been of assistance in roughly estimating the course of the bacteriemia. During the acute phase of the disease the early appearance of innumerable colonies is the usual finding. In the chronic phase when positive, the culture shows few or sometimes a single colony in 10 or more days.

The colonies of Brucella are clearly seen through the agar layer and are easily transferred for further study. With some experience one may differentiate them from contaminants, Salmonella and other organisms.

We consider that the ease with which positive cultures are detected and the reduction of bacteriological manipulation and material, plus the increased safety to the technician, compensate for the trouble taken in the preparation of the double medium. Furthermore, the technician in charge of identification of the organism receives only positive cultures and need not be warned about careless handling of the material. The possibilities of accidental infection are thereby reduced. The increase in the proportionate amounts of agar in the solid medium is necessary in order to prevent the deterioration of the agar layer. The rather high citrate content is intended not only to prevent clotting of the blood but also to reduce the phagocytic activity of polymorphonuclear leucocytes to a minimum. It is thought that the ease with which the Brucella colonies appear on the agar layer is due at least in part to the absence of plasma, thus reducing the bactericidal effect.

The 100 cc bottles have been found suitable for inoculation with 100 ml of blood. However, 200 or even 500 ml bottles may be used if one desires to culture larger amounts of blood.

Summary. A double medium for blood cultivation in brucellosis is described. The method saves time and material by avoiding transfers and affords increased safety against accidental infection to the personnel.

15718

Hematologic Effects of Pteroylglutamic Acid (Folic Acid) in Man.*

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Pteroylglutamic acid, commonly known as folic acid or *L. casei* factor, has been shown by several groups of investigators to be an effective hematopoietic substance in human macrocytic anemia.[1,2,3] Since 1945 when synthetic pteroylglutamic acid became available, 23 patients with macrocytic anemia have been treated with this material in our clinic. The findings obtained in 15 patients during the first few months of therapy have been reported.[4,5,6] Five patients have now been followed for approximately one year and 2 additional patients for over 6 months. In each instance, the clinical and hematological improvement which followed the administration of pteroylglutamic acid has been maintained.

Seventeen of the 23 patients studied were benefited by therapy. Hematologic findings at the initial and most recent visits to the clinic, and the amount and method of administration of pteroylglutamic acid are given in Table I. In all patients with pernicious anemia and nutritional macrocytic anemia there was a marked increase in the erythrocyte count, in hemoglobin and in the volume of packed red blood cells. In patients with sprue the blood picture was affected in only 3 of 5 cases. However, each patient improved in other respects including relief of glossitis and diarrhea, gain in weight, better absorption of nutrients from the intestinal tract as indicated by the glucose tolerance test, and diminution in steatorrhea. In one patient with cirrhosis of the liver there was a slight increase in the blood count after pteroylglutamic acid was administered while in 5 patients with macrocytic anemia of varied

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* This research was supported by grants from the Nutrition Foundation, Inc., and the United States Public Health Service. Pteroylglutamic acid was furnished through the courtesy of Lederle Laboratories.

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[4] Goldsmith, G. A., *Fed. Proc.*, 1946, **5**, 232.

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TABLE I.
Effect of Pteroylglutamic Acid on the Blood Picture in Macrocytic Anemia.

	Hematologic findings							Pteroylglutamic acid therapy		
	Initial			Final						
Sex Race	RBC	Hb	Hct	RBC	Hb	Hct	No. months observation	mg/day	Time	Route
				Pernicious Anemia.						
WF	1.7	6.2	21	4.2	13.0	40	12	15‡	21D	IM
								15	7M	O
								5	3M	O
CM	1.7	4.9	14	4.0	13.0	44	12	15	21D	IM
								0	6M	—
								5	5M	O
WM	3.5	10.5	36	5.0	14.9	46	7	120	5D	O
								30	14D	O
								5	6M	O
CM	1.0	3.3	10	3.5	11.2	41	2	5	2M	O
WF	1.4	6.0	21	4.2	13.2	42	3.5	10	3M	O
WF	2.1	8.2	23	4.6	14.5	47	3.5	10	3.5M	O
CM	1.5	4.2	16	4.0	10.3	39	2	5	2M	O
				Nutritional Macrocytic Anemia.						
WF	1.8	6.0	20	3.9	12.0	39	12	15‡	21D	IM
								15	3M	O
								30	1M	O
								5	6M	O
WF	2.3	7.0	22	4.1	13.9	44	11	15‡	21D	IM
								30	2M	O
								5	6M	O
WF	2.3	9.5	25	4.0	12.3	39	13	5-15†	5M	O
								30	3M	O
								5	5M	O
WF	2.3	8.4	29	4.4	11.5	40	8	40§	2M	IM
								100‡	7D	O
								5	5M	O
					Sprue.					
WF	3.5	11.5	39	3.7	10.2	35	3.5	50	21D	O
								20-40§	2M	IM
WF	3.6	11.3	38	3.9	11.9	39	2.5	30	2M	O
WM	2.1	7.3	27	4.0	12.5	39	3	5	3M	O
WM	3.0	10.0	36	3.4	11.0	37	1	20¶	1M	IM
WF	3.4	10.9	37	3.8	11.5	38	1	10	1M	O
WM*	3.9	10.7	38	4.4	12.6	41	1	20	14D	IM

IM—Intramuscular administration; O—Oral administration; D—Days; M—Months; Hct—Hematocrit-volume of packed erythrocytes %.

* Celiac disease.

† Therapy intermittent.

‡ Therapy omitted for 1-2 months after this period of treatment.

§ This dosage administered 3 times weekly.

¶ This dosage administered every 4 days.

etiology there was no appreciable change (2 had aplastic anemia, one each, anemia associated with regional ileitis and myxdema and one anemia of unknown origin).

The hematologic effects of pteroylglutamic acid closely resembled those obtained with liver extract. Within 2 to 6 days after therapy was instituted the percentage of reticulocytes in the peripheral blood increased, a maximum rise being attained in 5 to 14 days. When pteroylglutamic acid was given orally the increase in reticulocytes was similar to that which is usually observed when optimal amounts of liver extract are administered; when given parenterally the increase in reticulocytes was usually less than this (Fig. 1). During the first few days of treatment hemodilution occurred and edema was found on physical examination. The erythrocyte count, percentage of hemoglobin and the volume of packed red blood cells began to rise 7 to 10 days after therapy was instituted. The increase was rapid during the first month and then gradual until normal or slightly sub-

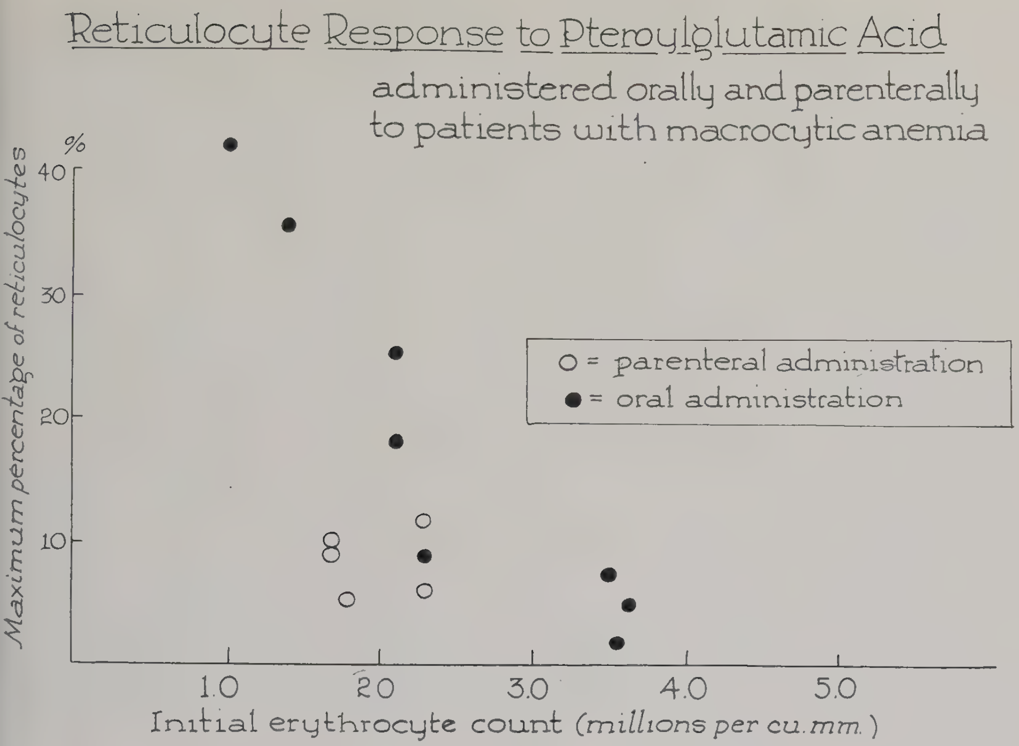

FIG. 1.
The amount of pteroylglutamic acid given parenterally was 15 mg or more daily; the oral dose in the four patients with a reticulocyte response above 15% was 5 to 10 mg daily.

ormal levels were reached. The monthly rythrocyte counts for 7 patients who have een followed for over 6 months are given in Table II. The maximum rise occurred by the hird month in most instances and this level as been maintained essentially unchanged. The count has remained below 4.5 million in a majority of the patients in spite of intensive treatment. This may indicate a difference between the effects of pteroylglutamic acid and of liver extract.

A dose of 5 mg of pteroylglutamic acid daily

TABLE II.
Erythrocyte Count in Patients with Macrocytic Anemia During Treatment with Pteroylglutamic Acid.

Sex Race	Initial count	Erythrocyte count (millions per mm^3) monthly intervals												
		1	2	3	4	5	6	7	8	9	10	11	12	13
		Pernicious Anemia.												
W.F.	1.7^{2}	3.2^{0}	3.3^{2}	4.0	3.9	4.0^{5}	4.3	3.9	4.5	4.5^{1}	3.4	3.6	4.2	
C.M.	1.7^{2}	3.3^{0}	3.5	4.1	4.2	3.0	2.5	2.3^{1}	3.4	3.5	4.3	4.2	4.0	
W.M.	3.4^{4}	4.2^{1}	5.8	6.0	4.4	4.5	4.7	5.0						
		Nutritional Macrocytic Anemia.												
W.F.	1.8^{2}	3.4^{0}	3.3^{2}	4.0	3.9	3.9^{3}	$4.2^{1,5}$	4.2	4.3	3.9	4.0		3.9	
W.F.	2.3^{2}	3.5^{0}	3.6	3.6^{3}	3.7	3.8^{1}	4.4	4.5	4.5	4.3	4.0	4.1		
W.F.*	2.3^{1}	2.9^{2}	3.0^{0}	3.1^{2}	3.4^{0}	3.9^{3}	4.0	4.1	4.2^{1}	4.0	4.0	3.8	4.0	4.0
W.F.	2.3^{6}	3.7	3.7^{7}	4.0^{1}	4.2		4.0	4.1	4.4					

* Therapy intermittent during first 5 months.

Amount of pteroylglutamic acid administered and changes in therapy are indicated by numerals as follows: (0) no therapy; (1) 5 mg daily; (2) 15 mg daily; (3) 30 mg daily; (4) 120 mg daily for 5 days then 30 mg daily for 2 weeks; (5) iron added to therapy; (6) 40 mg three times weekly; (7) 100 mg daily for 1 week then none for 1 month.

appeared to be approximately as effective as were larger amounts in producing reticulocytosis and an increase in erythrocyte count. The administration of 30 mg daily did not cause significant improvement over that seen with 15 mg a day (cases 4-5-6, Table II). However, the only patient (Case 3) whose red cell count reached 5 million was given very large amounts of pteroylglutamic acid initially. The 7 patients who have been followed for prolonged periods have received only 5 mg daily for the past 3 to 6 months. During this time the erythrocyte counts have remained essentially constant. This dosage seemed to be sufficient and may be more than the minimal amount needed for maintenance.

The initial leukocyte count was low (less than 3500 per mm³) in 6 patients included in this study. In each instance there was a return to normal when pteroylglutamic acid was administered and the percentage of granulocytes increased. The rise in white cells began during the first week of therapy and reached a maximum in 1 to 3 weeks.

The bone marrow was studied in most of the patients treated with pteroylglutamic acid and showed a rapid return to normal. In pernicious anemia the marrow is hyperplastic, megaloblasts being the predominant cell. In one patient in whom serial studies were undertaken, megaloblasts had decreased in number at the end of 5 days and were rarely observed at the end of 2 weeks. In patients with nutritional macrocytic anemia the marrow was at times hyperplastic and at others hypoplastic. The predominant cell was the megaloblast in some instances, the normoblast in others. Regardless of the findings prior to therapy, the bone marrow became normal within 4 to 6 weeks.

Conclusion. The administration of pteroylglutamic acid was followed by definite improvement in 17 of 23 cases of human macrocytic anemia. The hematologic effects of pteroylglutamic acid closely resembled those of liver extract but differed in that oral administration was equally or more effective than parenteral and that restoration of the blood picture to normal was often incomplete.

15719 P

Antibiotic Activity of Certain Molds Against *Brucella*.*

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Although a number of mold antibiotics have been described,[1] few reports indicate activity against *Brucella spp.*[2,3] The ineffectiveness of penicillin against bacteria of this group was early recognized,[4] and this observation has received repeated confirmation. Despite the demonstration of inhibition of Brucella by high concentrations of penicillin,[5,6] resistance of these organisms is so great that the drug has been successfully used in concentrations of 2-5 Oxford units per ml for selective isolation of Brucella from material containing other more sensitive bacteria.[6]

Experimental. In a preliminary study 202 mold cultures,‡ including strains of Aspergillus, Penicillium, Absidia, Rhizopus, Mucor, Chaetomium, Trichoderma, Sporotrichum,

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* Aided by a grant from Swift and Company.

† The early part of this study was made at The Brucellosis Research Laboratory, Clayton Foundation, The University of Texas.

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‡ Cultures were kindly made available by Dr. M. B. Morrow, Department of Botany and Bacteriology, The University of Texas.

TABLE I.

Antibiotic Activity of Certain Molds Against Brucella and Staphylococcus.

Mold culture	Source of mold	Activity of Filtrates*	
		Brucella abortus 295	*Staphylococcus aureus* 209
Aspergillus terreus No. 1	Stock collection	+	0
” ” No. 2	” ”	+	0
” ” No. 3	” ”	+	0
” ” No. 4	” ”	+	+
” *sydowi* No. 1	” ”	0	+
” *nidulans* No. 1	” ”	0	+
Penicillium carminoviolaceum No. 1	” ”	+	+
” *sartoryi* No. 1	” ”	+	+
” *chrysogenum*	” ”	0	+
” sp., SD-1	Soil†	+	+
” ” SD-21	”	+	+
Aspergillus glaucus, SD-3	”	+	+
” *versicolor*, SD-5	”	0	+
” *terreus*, SD-16	”	+	+
” ” SD-17	”	+	+
” *fumigatus*, SD-19	”	0	+
” *flavipes*, SD-16	”	+	+
” *humicola*, SD-23	”	+	0
” *versicolor*, SD-7	”	0	+

* Filtrates of mold cultures in Czapek-Dox medium were tested against 0.1 ml of 1:10 dilution of bacterial culture in 100 ml tryptose—0.1% dextrose agar. + = inhibition; 0 = no inhibition.

† Freshly isolated from lot inclosing Brucella-infected cattle.

ullularia, Hormodendrum, Curvularia, Hel- inthosporium, Stemphylium, Alternaria, pondylocladium and Fusarium, were tested r inhibition of *Br. abortus* 295 (Huddleson) nd *Staph. aureus*. Molds were grown at oom temperature in a tryptose-0.1% dextrose roth, pH 7.3. Metabolite samples, diluted Locke's solution, were tested for activity ter 6, 9, 12, 19, and 26 days against *Staph. ureus* and *Br. abortus* by a modified cup ethod,[7] using 20 ml of tryptose agar seeded ith 0.1 ml of a 24-hr culture of the test acteria. One culture of *Penicillium spinu- sum* inhibited growth of *Staph. aureus* and ne *Aspergillus flavus* inhibited both *Staph. ureus* and *Br. abortus* by this method.

Thirty-two strains of Penicillium and Asper- llus found to be inactive in the above tests ere grown in modified Czapek-Dox medium[8] nd the undiluted filtrates were assayed gainst *Br. abortus* 295 and *Staph. aureus* 209, using 0.1 ml of 1:10 dilution of bacterial culture in 100 ml tryptose agar. Filtrates of cultures of nine of the molds were active against one or both of the bacteria. Of 24 molds isolated from soil of an inclosure in which Brucella-infected cattle were kept, 10 were found to be active by the same method (Table I). In some instances activity was slight or inconsistent.

Mold SD-17, tentatively classified as *Aspergillus terreus,* was particularly active against Brucella. On Czapek-Dox medium this culture produces a tan-colored colony which is brown on the reverse side; microscopically the heads are columnar, and the conidia smooth and globose. Further studies were made on cultures of this mold grown in Czapek-Dox broth. Active filtrates of a 14-day culture were wine-colored. The active material is found in the ether soluble fraction, is heat stable (boiling 10 minutes), and after partial purification inhibits *Br. abortus in vitro* in a dilution of 1:64,000.

Since citrinin is produced by a related species,[9] comparative studies have been made with crystalline citrinin supplied by Dr.

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Timonin. The antibacterial spectra of the two substances are similar and the activity of mold SD-17 may be due to citrinin. The active material produced by mold SD-17 has not been obtained in a crystalline form, and its identity has not been determined.

Summary. Filtrates of 13 mold cultures were found to possess antibiotic activity against *Br. abortus in vitro.* The active material from one of these molds, tentatively identified as *Aspergillus terreus,* after partial purification, inhibited growth of *Br. abortus* in a dilution of 1:64,000.

15720

Effect of Para-Aminohippurate on Mannitol Determinations by the Periodate-Iodide-Thiosulfate Method.*

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In using the combination of mannitol[1] and para-aminohippurate[2] in renal function studies we noticed discrepancies which suggested to us that the presence of para-aminohippurate (PAH) might be affecting the chemical analyses for mannitol. Therefore, we prepared solutions containing no mannitol but in which known amounts of free para-aminohippuric acid (PAHA) were added in varying concentrations to water and to sugar- and protein-free filtrates of human plasma and of urine. These solutions were then analyzed by the technic used for estimation of mannitol.[1] This involves oxidation of mannitol with a known excess of KIO_4 and determination, by titration with thiosulfate, of the remaining KIO_4 together with the KIO_3 produced in the reaction. Fig. 1 shows the results of these analyses. It can be seen that for every mg % of free PAH present, there is an apparent mannitol concentration of from 0.25 to 0.30 mg %.

In order to illustrate the extent to which this error can be expected to influence the various data of clinical interest, in a representative renal work-up with mannitol and PAH, we have prepared Table I. The data are calculated for an hypothetical patient based on a plasma mannitol level of 1 mg per cc throughout; a plasma PAH level of 0.02 mg per cc during the "plasma flow period" and 0.75 mg per cc during the "Tm period." The glomerular filtration rate is taken as 130 cc per min., the effective renal plasma flow as 670 cc per min., and the Tm_{PAH} as 75 mg per min. It is assumed that there is a false elevation of 0.27 mg % in mannitol levels for each mg % of PAH present.

One study in the literature[3] reports the glomerular filtration rate (mannitol) on each of 2 different days (4 in 2 cases) in 23 patients. In each instance, the mannitol clearance was done during a Tm determination with PAH on one day and during a diodrast Tm on the other day. In the 25 pairs, the mannitol clearance was higher on the PAH day in 21 cases and lower in 4 instances. The mean C_M was 9.2% higher when PAH was present at Tm levels than on days when diodrast was used. This agrees well with the 10% expected error listed in Table I.

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* The expenses of this investigation were defrayed in part by a grant from the Life Insurance Medical Research Fund to the Department of Pharmacology, University of Pennsylvania.

† Research Fellow in Pharmacology and Fellow in the Harrison Department of Surgical Research.

‡ Research Fellow in Pharmacology.

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[3] Chasis, H., Redish, J., Goldring, W., Ranges, H. A., and Smith, H. W., *J. Clin. Invest.,* 1945, **24**, 583.

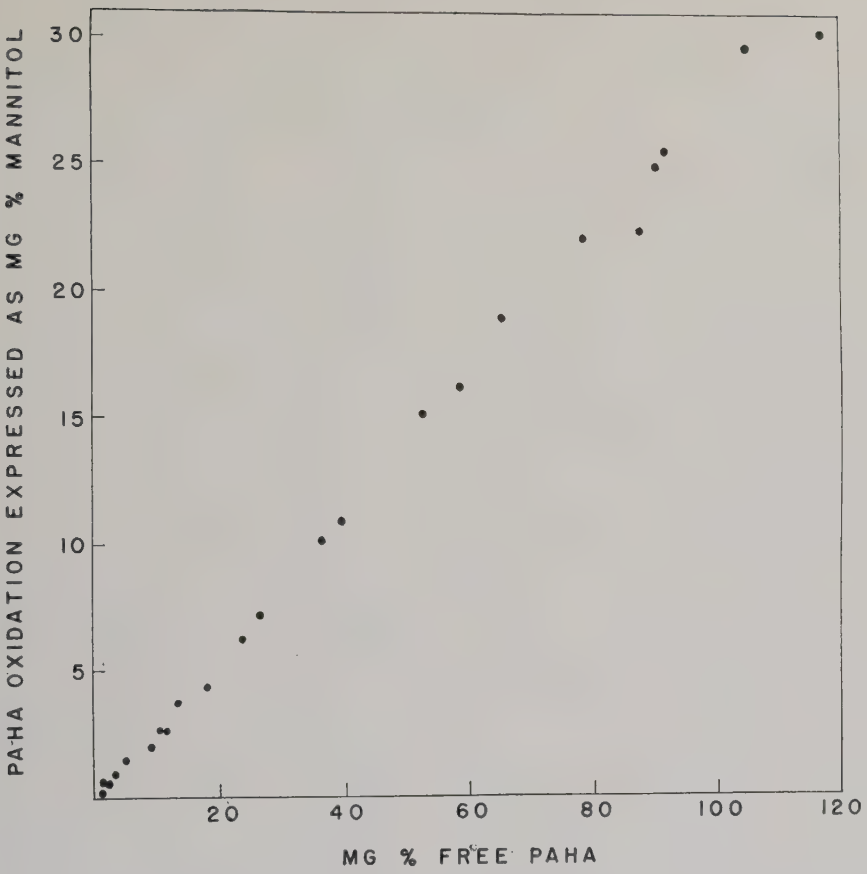

Fig. 1.

Para aminohippuric acid solutions analyzed by the unmodified method used for mannitol determination.

n the same paper an attempt was made to stablish the Tm_{PAH} for normal subjects. One eries of 10 cases gave a mean Tm_{PAH} of 7.6 using mannitol to determine glomerular ltration rate and another series of 21 cases gave a mean of 82.2 using inulin for filtration rate estimation. It would seem that the normal Tm_{PAH} is probably higher than 77.5 (the average for the 2 series) and the standard deviation is less than 12.9, as there

TABLE I.

rrors in Clinical Data to Be Expected from the Unmodified Mannitol Method When Used in the Presence of PAH.

		Apparent		% error	
	Correct	During C_{PAH}	During Tm_{PAH}	During C_{PAH}	During Tm_{PAH}
annitol excretion (U_MV) mg/min	130	134	173	3	30
lasma mannitol level (P_M) mg/cc	1	1.006	1.2	0.6	20
lomerular filtration rate (C_M) cc/min	130	133	143	2	10
ax. tubular excretory capacity (Tm_{PAH}) mg/min	75		67		10
iltration fraction (C_M/C_{PAH})	0.194	0.20		3	
iltration rate per functioning unit (C_M/Tm_{PAH})	1.9		2.6		40
ffective renal plasma flow per functioning unit (C_{PAH}/Tm_{PAH})	8.9		10		10

See text for plasma levels, etc., assumed in preparation of this table.

TABLE II.
Mannitol Recoveries by the Modified Method.

Substance to which mannitol and PAH were added	PAH mg % by weight	Mannitol mg % by weight	Mannitol mg % by analysis	Error %
Water	0	10.0	9.92	—1
,,	8.72	8.35	8.28	—1
,,	8.72	8.35	8.10	—3
,,	10.46	10.02	10.06	+0.04
,,	10.46	10.02	10.17	+2
Urine filtrate	10.46	10.02	9.92	—1
Plasma ,,	10.46	10.02	9.87	—1
,,	77.0	96.6	99.6	+3
,,	77.0	96.6	97.7	+1

Each recovery was run in duplicate and the analytic results are the averages. Samples with like concentrations represent separate dilutions in different volumetric flasks, but from the same stock solutions.

reported.

Unfortunately the oxidation of PAH is not sufficiently quantitative to enable accurate correction of mannitol figures on the basis of that reaction.

Acetylation of PAH was found to prevent its oxidation and it was possible to accomplish this without detectable acetylation of mannitol taking place.

Hydrolysis of acetylated PAH to free PAH occurs when it is boiled in the KIO_4-H_2SO_4 solution usually used, but this can be avoided by replacing the sulfuric acid with acetic acid in the periodate solution. The sulfate which persists from the $CdSO_4$-H_2SO_4 solution used for precipitation of proteins does not seem to affect results.

Method. A modification of the periodate-iodide-thiosulfate method[1] which we have found to be satisfactory is as follows: 1. Glucose is removed from plasma and urine samples by yeast fermentation and protein is precipitated with cadmium sulfate. 2. Duplicate 2 cc samples of filtrate are pipetted into pyrex ignition tubes. 3. Roughly 1 cc of acetic anhydride is added from a bacterial pipette and the tubes are shaken several times during the next 5 or 10 minutes. 4. Exactly 5 cc of KIO_4-acetic acid solution (0.06 Gm KIO_4 dissolved in 100 cc of 20% acetic acid) is pipetted into the tubes. The tubes are covered with glass tears and boiled in a water bath for 20 minutes. 5. After cooling, about 0.5 cc of a 10% KI solution is added and the liberated iodine titrated immediately with sodium thiosulfate, using a starch indicator.

Recoveries of mannitol determined by this method in the presence of PAH are shown in Table II.

Summary. 1. The periodate-iodide-thiosulfate technic for mannitol determinations requires revision when para-aminohippurate is present. For each mg % of free PAH present, the mannitol recovery will be falsely elevated by from 0.25 to 0.30 mg %. 2. A modification of the technic is presented by which mannitol can be determined in the presence of PAH.§

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§ A method for mannitol estimations has been advanced[4,5] which measures quantitatively one of the products of the reaction between mannitol and periodate. The presence of PAH should make no difference in such a procedure if a sufficient excess of periodate is used.

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15721

Factors Not Synthesized by Yeast in the Presence of Cyanide.

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This study was undertaken as a result of difficulties encountered in growing yeast in a synthetic medium in the presence of potassium cyanide.[1] Other investigators[2,3] have reported no difficulty in culturing *Saccharomyces cerevisiae* in a medium containing the same concentration of cyanide, but their media contained malt extract or yeast extract. Presumably the extracts supply one or more substances which the yeast is unable to synthesize with sufficient rapidity in the presence of cyanide. Direct experiments supported this view.

A method for quantitative study of the active principle or principles present in yeast extract was devised, the procedure being patterned after that used in the assay method for biotin.[4] Initially, the only modification in the culture medium was the addition of a sufficient amount of potassium cyanide to give a concentration of 0.001 *M*. Later, pantothenic acid (2 mg per liter) was substituted for β-alanine, and cysteine (20 mg per liter) was added as a supplement. The standard of activity was Difco yeast extract which was added over a range of 0 to 10 mg. The test organism was *Saccharomyces cerevisiae,* F.B., and the extent of growth was measured turbidimetrically after an incubation period of 15 to 16 hours at 30°C. Table I shows the growth response of F.B. yeast to various levels of yeast extract under test conditions.

A large number of known compounds including the B vitamins, most of the familiar amino acids, various saturated, unsaturated, and hydroxy acids, certain purines and pyrimidines, adenosine, adenylic acid, glutathione, sodium thioglycolate, and ascorbic acid were found to be inactive. Only cysteine and methionine showed any activity and neither of these produced an effect comparable with that of yeast extract.

None of the various source materials tested was as active as yeast extract. A commercial hydrolysate of casein (casamino acids) was found to resemble methionine in effect, but by measuring the growth stimulation of mixtures of this preparation and yeast extract and of methionine and yeast extract it was established that the activity of the hydrolysate did not depend solely on its content of methionine.

Substitution of pantothenic acid for β-alanine in the test medium resulted in an enhanced growth stimulation by casamino acids. A concentration of 10 γ per tube of the vitamin was as effective as 100 γ and was considerably more effective than 100 γ of β-alanine. If only 2 factors, pantothenic acid (present in yeast extract in nonlimiting concentration) and some other substance (contained in both yeast extract and casamino acids), were involved in the reversal of cyanide inhibition of growth, the growth stimulation produced by adding yeast extract to casamino acids in a pantothenic acid medium should represent an additive effect.

TABLE I.
Growth Response of F.B. Yeast to Yeast Extract in Cyanide Medium.

Yeast extract added per tube, mg	Galvanometer reading
0	5
0.2	11
0.5	22.5
1.0	32
5.0	62
10.0	81

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* Present address: Foods and Nutrition Department, Iowa State College, Ames, Iowa.

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TABLE II.

Anti-Cyanide Activity of Yeast Extract and Casamino Acids, Separately and Combined, in Media Containing β-Alanine or Pantothenic Acid.

Amt of yeast extract added, mg	Extent of growth in various media							
	β-alanine medium		β-alanine medium + casamino acids*		Pantothenic acid medium		Pantothenic acid medium + casamino acids*	
	Galvanometer readings	Apparent mg units†	Galvanometer readings	Apparent mg units	Galvanometer readings	Apparent mg units	Galvanometer readings	Apparent mg units
0	6	0	23	0.65	6	0	40	2.0
0.5	21	0.5	49	3.0	22	0.6	54	3.8
1.0	28	1.0	56	4.4	28	1.0	59	5.0
5.0	59	5.0	69	>5	59	5.0	73	>5

* The concentration of casamino acids was 10 mg per tube.

† The response to yeast extract alone in the β-alanine medium was used as standard.

The results in Table II show this not to be the case. It appeared, therefore, that at least 2 factors other than pantothenic acid must be involved. For all subsequent tests pantothenic acid was substituted for β-alanine in the culture medium.

The growth-promoting effect of yeast extract was improved by including in the medium any one of several reducing agents (ascorbic acid, sodium thioglycolate, glutathione, sodium hydrosulfite or cysteine). The optimum growth stimulation occurred in the medium containing 20 mg per liter of cysteine. For all of the experiments which followed this observation, the cyanide medium was supplemented with cysteine.

Further investigation provided evidence confirming the supposition that at least 2 substances other than pantothenic acid were responsible for the growth-promoting activity of yeast extract in a cyanide medium. These factors could be separated by adsorption with Darco G-60. The active material in the unadsorbed fraction was designated as the filtrate principle while that in the adsorbed portion which was eluted with an aqueous mixture of alcohol (40%) and ammonia (10%) was termed the eluate principle.

Although attempts to identify the filtrate and eluate principles with known compounds were unsuccessful, some information concerning their properties was obtained. Both principles are soluble in 70% ethyl alcohol and are unaffected by autoclaving in the presence of normal solutions of acid and base or by exposure to light. The filtrate principle is partially inactivated by treatment with nitrous acid and completely inactivated by hydrogen peroxide while the eluate principle is virtually unaffected by both of these reagents.

The results in Table III show other differences between the filtrate and eluate principles. The similar behavior of the filtrate principle and casamino acids made it seem likely that the filtrate principle was an amino acid. However, of a number of amino acids tested, all were inactive except norleucine which was inhibitory. The possibilities remain that the filtrate principle is an amino

TABLE III.
Growth Promoting Effect of Various Materials in Cyanide Medium.

Material tested	Apparent potency
Yeast extract (standard of reference)	1.0
Filtrate preparation	0.18
Eluate preparation	0.07
Casamino acids	0.20
Yeast extract (1 mg) + filtrate (1 mg equivalent)	2.1
Yeast extract (1 mg) + eluate (1 mg equivalent)	1.0
Yeast extract (1 mg) + casamino acids (1 mg)	2.0
Filtrate + eluate (equivalent amts of each)	1.0
Filtrate (1 mg equivalent) + casamino acids (1 mg)	0.40
Eluate (1 mg equivalent) + casamino acids (1 mg)	1.1
Filtrate + nicotinic acid (10 γ per tube)	0.27
Filtrate + folic acid (20γ per tube)	0.22
Filtrate + nicotinic acid (10 γ) + folic acid (20 γ)	0.36

acid other than the ones tested or that its effect is a composite one due to several amino acids.

Even though nicotinic acid (or its amide) and folic acid were found to be somewhat stimulatory, neither individually nor variously combined did they behave in the same manner as did the eluate principle.

By repeated adsorption and elution from charcoal, a preparation was obtained which was approximately 8 times as active as that resulting from the initial treatment of yeast extract. A 6-fold concentration of the eluate principle was achieved by refluxing yeast extract with 95% ethyl alcohol and treating the soluble fraction once with Darco G-60.

Evidence indicating that the activity of eluate preparations does not depend on a single substance was derived by subjecting the principle to electrolysis at high voltage in the electrical transport apparatus described by Williams and Truesdail.[5] The active principle was not concentrated in a single cell but was distributed throughout the 4-cell system employed.

Summary. Yeast extract has been shown to reverse the inhibition of yeast growth caused by cyanide. No known compound was found which duplicated the action of yeast extract, although methionine and cysteine had some "anti-cyanide" activity. Excess pantothenic acid had a beneficial effect on growth under certain conditions.

It has been demonstrated that the action of yeast extract depends on at least 2 substances which can be separated by charcoal adsorption. Indirect evidence indicates that the activity of both filtrate and eluate principles is due to more than a single substance.

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Effects of Various Anesthetic Agents on the Blood Pressure of the White Rat.*

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In an investigation involving the role of the autonomic nerves in cardiovascular disorders it was found desirable to measure the blood pressure repeatedly in rats without injury to the animals and to determine the effect of anesthetic agents on the blood pressure. A survey of the literature showed that the apparatus used was varied and that de-

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* This work was supported by a grant from the Life Insurance Medical Research Fund.

terminations of blood pressure were in many cases inconsistent. In some instances no mention was made of the anesthesia used.

The observations of Page and Reed,[1] Williams, Harrison and Grollman,[2] Kempf and Page,[3] and Medoff and Bongiovanni[4] are somewhat in agreement. All of their blood pressure determinations fall within a range of 100 to 140 mm Hg. Duncan, Hyman and Chambers[5] reported blood pressure determinations from 90 to 140 in unanesthetized rats and from 70 to 120 in anesthetized animals. The studies of Telford, Swegart and Schoern[6] are in agreement with those of the above workers, but in addition they found, in animals one year older, a blood pressure range between 84 and 95. Proskauer, Neumann and Graef[7] reported normal blood pressure readings of 60 to 95 mm Hg. Duncan *et al.*[5] found a decrease in blood pressure within a range of 70 to 120 in anesthetized animals and Byrom and Wilson,[8] using ether, obtained blood pressures from 72 to 132 mm Hg.

Corcoran and Page,[9] studying the effects of pentobarbital-sodium on the blood pressure of dogs, reported an increase in blood pressure, whereas Mylon, Winternitz and de Suto-Nogy[10] found that nembutal simulates a condition like shock causing a lowering in blood pressure. Grimson, Kernodle and Hill,[11] studying the effects of various anesthetics on the blood pressure of dogs, obtained wholly inconsistent results.

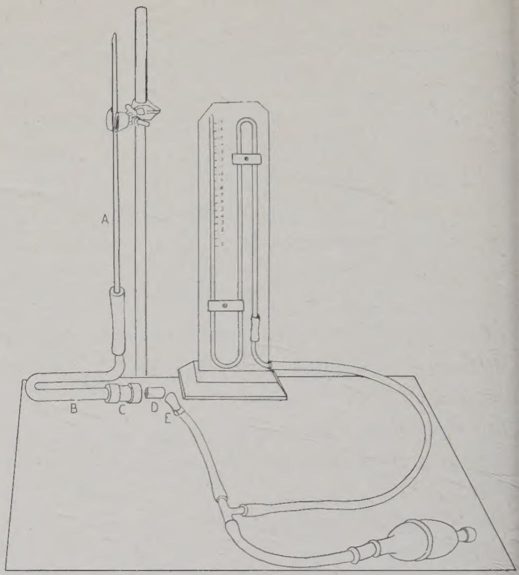

FIG. 1.
Blood pressure apparatus: A, 0.2 mm pipette; B, plethysmograph; C, gland, D, metal cuff; E, rubber pressure cuff.

In a study of spinal anesthesia in a large number of human cases Koster[12] found that it lowered the blood pressure which he believes due to a decrease in cardiac output.

Methods. In the present study, 40 female white rats of the Wistar Strain weighing 135 to 200 g were used. The room temperature at the time of the determinations was kept at 25 ± 2°C. A modification of the tail plethysmograph method of Byrom and Wilson[8] was used to determine blood pressure. It was found that the pressure cuff described by Griffith and Jeffers[13] was most suitable. The plethysmograph consisted of a piece of bent glass tubing with a bore of 1 cm and a 0.2 mm bacteriological pipette (Fig. 1).

Unanesthetized rats were wrapped in a Turkish towel. In a short time they became quiescent and did not appear to be disturbed by the procedure. From 4 to 6 readings were

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[2] Williams, J. R., Harrison, T. R., and Grollman, A., *J. Clin. Invest.*, 1939, **18**, 373.

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[12] Koster, H., *Arch. Surg.*, 1942, **45**, 596.

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TABLE I.
Effects of Various Anesthetic Agents on Systolic Blood Pressure of the White Rat.

	Mean, mm Hg.	Stand. dev., mm Hg.	Range, mm Hg.
Unanesthetized	124.8	7.54	102-140
Nembutal	104.1	7.78	86-115
Amytal	103.5	6.01	94-112
Ether (shallow)	117.0	4.62	110-126
Ether (deep)	104.0	4.91	95-110
Urethane	78.4	3.75	72- 84
Morphine	63.9	1.31	62- 70

made on each animal. If there was marked variation in the readings they were discarded and others were taken at a later time. The anesthetics used were given in the following dosages: pentobarbital-sodium (nembutal), 4 mg/100 g; sodium isoamylethyl barbiturate (amytal), 4 mg/100 g; ethyl carbamate (urethane), 20 mg/100 g; and morphine sulphate, 10 mg/100 g. Ether was applied by sponge. The other agents were all administered by intraperitoneal injection. In order to test the apparatus for high blood pressure some rats were made hypertensive by a high tryosine diet and others were injected with adrenalin. All the tabulations obtained from the observations were treated to a statistical analysis.

Results. The experimental data obtained are summarized in Table I. In the unanesthetized group only one rat had a blood pressure as low as 102 mm Hg and only one had a pressure as high as 140 mm Hg. The remaining animals were in a range between 113 and 132. Animals of 2 groups, one with an average weight of 140 g and the other with an average weight of 200 g showed no significant differences in their blood pressures.

Rats that had been placed on a 10% tyrosine diet for 2 weeks preceding their blood pressure determination showed a mean arterial systolic pressure of 168 mm Hg. Those injected with 0.5 cc of 1/1000 adrenalin showed a rise in the systolic pressure from the normal level to 180 mm Hg. The pressure then increased at a slower rate until it reached a peak of 210 mm Hg after which it receded to a normal level.

When ether was used there was considerable variation in the blood pressure determinations in individual rats. There was a marked decrease in blood pressure during the application of the ether sponge or shortly after, a rise in pressure when the effects of the ether subsided. Experiments on several animals indicate that there is a direct correlation between the decrease in blood pressure and the depth of the anesthesia.

The markedly lowered pressures that were obtained with the use of morphine and urethane may be explained on the basis of the vasodilator effects of these agents. The barbiturates (nembutal and amytal), according to Roth,[14] act as cardiac depressants in the white rat which may account, to some extent, for the decrease in blood pressure.

Summary. The method of Byrom and Wilson as modified for this study is well adapted for the determination of blood pressure in the rat. The mean arterial systolic pressure in the unanesthetized animal was found to be 124.8 mm Hg. The mean pressures obtained by the use of anesthetic agents were as follows: nembutal—104.1, amytal—103.5, ether (shallow)—117, ether (deep)—104, urethane—78.4, morphine—63.9 mm Hg.

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